昭和 32 年 7 月 9 日 第三種郵便物 認可
第 48 巻 第 2 号 昭和 35 年 8 月 25 日発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 48, No. 2, August 25, 1960

Vol. 48, No. 2 **August, 1960**

THE JOURNAL

OF

BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutland, Vt.
U.S.A.

Maruzen Co., Ltd.
Tokyo
Japan

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PRINTED IN JAPAN

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

A METHOD FOR THE MICRODETERMINATION OF LOWER ALIPHATIC FATTY ACIDS BY PAPER CHROMATOGRAPHY AND ITS APPLICATION TO STUDY OF THE METABOLISM OF ASCARIS LUMBRICOIDES VAR. SUIS*

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(Received for publication, November 12, 1959)

Investigations in this laboratory, involving analysis of mixtures of lower fatty acids in the excretory fluid and in the perienteric fluids of a parasite, *Ascaris lumbricoides* var. *suis*, indicated the need for a method more convenient than those at present available.

Elseden (*1*), Moyle *et al.* (*2*) and many other investigators used the silica-gel partition column chromatography for analysis of the mixed lower fatty acids and Bueding (*3*) succeeded in identification of some products of *Ascaris* using celite as adsorbent. However, this method is tedious and not convenient for analyses of many samples at the same time. Fink *et al.* (*4*) converted volatile and non-volatile acids to the corresponding hydroxamates, and then separated them by paper chromatography. This technique has been developed and employed by some other investigators.

Although these hydroxamates were clearly separated on paper chromatogram, the color intensity of their complex with ferric ion was so week that quantitative determination of micromole level of the substance was difficult. This difficulty led the author to develope a more sensitive colorimetric method based on the Feigl's principle; that is, the separation of fatty acids as hydroxamates on paper chromatogram and the colorimetric determination of the hydroxamates by converting to azo-dyestuff with α-naphtylamine (*5*).

In this paper, the procedures of this new method and its application to study of the metabolism of *Ascaris* are reported.

MATERIALS AND METHODS

Solvent Systems—Two solvent systems were employed; (a) redistilled n-butanol saturated with an equal volume of aqueous 3 N acetic acid, and (b) redistilled benzene saturated with an equal volume of 60 per cent formic acid.

α-Naphtylamine and hydroxylamine HCl were recrystallized from hexane and

* A brief account of this work was presented at the 15th and 30th Meeting of the Pharmacological Society held in Tokyo on 17 November, 1956 and 5 April, 1957 respectively.

methanol-HCl solution, respectively.

Nitrosomethylurea was prepared from acetamide according to the method of Amstutz *et al.* (*6*) and ether solution of diazomethan was obtained by decomposition of nitrosomethylurea with alkali.

Benzohydroxamic acid was synthesized according to Hauser *et al.* (*7*).

All other solvents and acids used were commercial products.

Animal—Ascaris was obtained from the Sibaura Slaughter House (Tokyo). After washing it in warm saline water, each specimen was transfered into 200 ml. of Locke-Ringer solution containing 10 units of penicillin, 50 μg. of streptomycin and 20 μg. of dehydroacetic acid per ml. After 24 hours the perienteric fluid and the medium in which *Ascaris* had been incubated were analyzed. Usually the perienteric fluid and the media of ten specimens were used in an experiment.

Preparation of Hydroxamic Acids and Chromatography—The sample to be tested was extracted with ether and ethereal diazomethane was added to the ether extract until evolution of nitrogen gas ceased and a persistant yellow color developed. This solution was allowed to stand for 15 minutes at room temperature. To this solution, 2 ml. of neutral methanolic hydroxylamine solution (2 *N* $NH_2OH \cdot HCl$ solution neutralized by addition of concentrated KOH solution, mixed just before use) was added and this mixture was reflexed for 30 minutes at 90° in a water bath. After cooling, the solution, added with 2 drops of concentrated HCl, was filtered to remove salts and brought to an appropriate volume with ethanol. Then 0.05 ml. aliquots of this ethanolic solution, containing 20 μg. or more of fatty acid, was applied to Toyo filter paper No. 53 (40×40 cm.) in an area of 0.5×3 cm. In some instance, multiple spotting may be necessary to provide a sufficient amount of hydroxamate for measurement. For multiple spotting, it is absolutely necessary that the spot should be completely dry before superposing any additional amount of the solution.

The paper was developed by ascending method for 10–15 hours. Butanol system was used for separating C_1-C_3 acids, and benzene system for C_3-C_9 acids.

Quantitative Determination of Hydroxamic Acids—After chromatography, spot areas of each hydroxamic acid were cut out of the developed paper strips and eluted with 10 ml. of 0.5 per cent sodium acetate; 0.2 ml. of 1 per cent sulfanilic acid in 25 per cent (v/v) aqueous acetic acid was then added to the eluate and followed by two drops of 0.1 *N* iodine solution in glacial acetic acid. The mixture was left to stand for a few minutes. Any excess free iodine was removed by adding 0.1 *N* sodium thiosulfate previous to the addition of a drop of 0.3 per cent α-naphthylamine in 30 per cent (v/v) acetic acid. Finally, 0.1 *M* acetate-HCl buffer (pH 3) was added to make up a total volume of 20 ml. Optical density of the solution was measured after 10 min. with a photometer at 515 mμ in 1 cm. cells.

RESULTS

The R_f values of the hydroxamates of authentic lower fatty acids, from formic (C_1) to pelargonic acid (C_9), are shown in Table I. All the acids tested were separated by combined use of the two solvent systems, except caprylic acid (C_8) which was inseparable from pelargonic acid as illustrated in Fig. 1. Considerable tailing was observed when the benzene-formic acid system was used in higher room temparature.

Absorption spectrum of this dyestuff (p-benzenesulfonyl-azo-α-naphthylamine) is illustrated in Fig. 2, which shows a absorption maximum at 515

mμ, but the extinction is dependent on pH of that solvent; the maximum extinction was observed at pH 2.9 and decreased both in neutral and more

TABLE I

Rf Values of Aliphatic Hydroxamic Acids

Acid	R_f (BuOH : AcOH : H_2O = 4 : 1 : 1)	R_f (Benzene : Formic acid = 1 : 1)
Formic	0.23	0.02
Acetic	0.49	0.04
Propionic	0.70	0.10
Butyric	0.89	0.18
n-Valeric	0.83	0.35
iso-Valeric	0.83	0.23
Caproic	0.84	0.52
Caprylic	0.86	0.90
Pelargonic	0.87	0.94

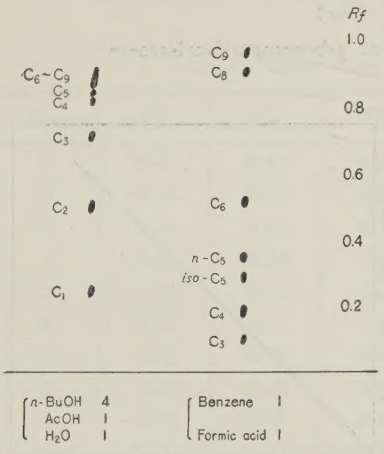


FIG. 1. Paper chromatogram of mixture of aliphatic hydroxamic acids.

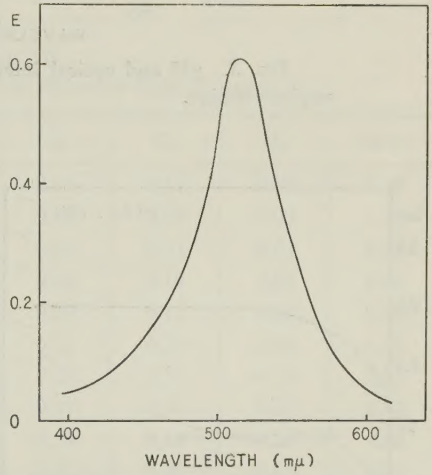


FIG. 2. Absorption spectrum of *p*-benzenesulfonyl-azo-α-naphthyl-amine.

acid side (Fig. 3). Therefore, the test solution was calibrated at pH 3 with 0.1 *M* acetate-HCl buffer.

Fig. 4 shows the stability of this red azo-dyestuff in buffered solution. Although the intensity increased rapidly by the addition of α-naphthylamine, this maximum intensity was obtained 10 min. after the addition of α-naphthylamine and the intensity remained constant for 30 min. at room

temperature. The heat treatment of the solution for 1 min. at 90° halved the time required for maximum color development.

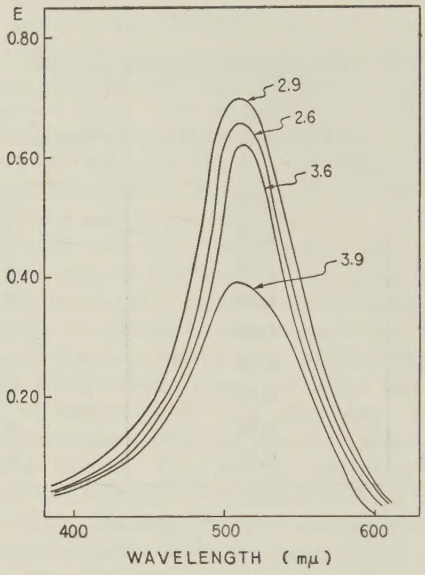


FIG. 3. pH and optical intensity of *p*-benzenesulfonyl-azo-α-naphthylamine.

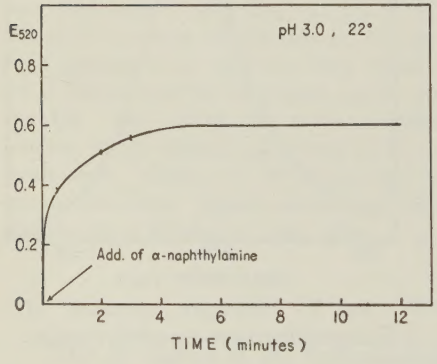


FIG. 4. Stability of *p*-benzenesulfonyl-azo-α-naphthylamine in acetate buffer.

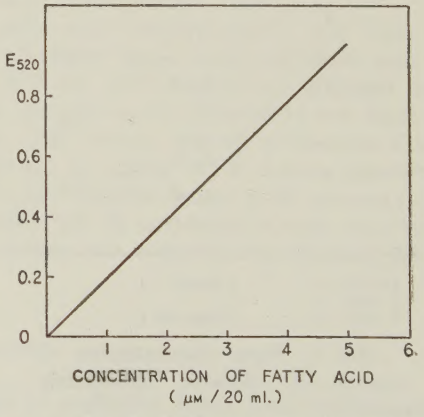


FIG. 5. Calibration curve of acyl-hydroxamic acids by the nitrite reaction.

Concentration curves obtained with lower aliphatic fatty acids are given in Fig. 5. The lower limit of this method is 0.5 μg. per ml. of fatty acid and the optical density was strictly proportional to the concentration of the fatty acid up to a density of 0.8 corresponding to 4 μM of fatty acid per 20 ml.

and the concentration curve of fatty acids thus obtained agreed with that of synthesized crystalline benzohydroxamic acid. Their recovery rate ranged between 92 and 97 per cent and no difference was observed between the two solvent systems (Table II).

TABLE II

Recovery of Fatty Acids from Known Mixtures

Acid	Solvent A (BuOH : AcOH : H_2O 4 : 1 : 1)	Solvent B (Benzene : Formic acid 1 : 1)
	%	%
Formic	0.92	
Acetic	0.95	
Propionic	0.94	
Butyric	0.97	0.94
n-Valeric	0.92	0.96
iso-Valeric		0.92
Caproic		0.93
Caprylic		0.92
Peralgonic		0.95

TABLE III

Contents of Lower Fatty Acids in Perienteric Fluid of Ascaris

Days	C_1	C_2	C_3	C_4	C_5	C_6	Total
1	0.72	0.17	0.24	0.14	0.12	0.08	1.47
2	0.73	0.05	0.23	0.05	0.04	0.03	1.13
3	0.55	0.09	0.01	0.06	0.01	0.01	0.73
4	0.50	0.12	0.01	0.08	0.12	0.03	0.86
5	0.44	0.11	0.01	0.09	0.12	0.03	0.80
6	0.73	0.15	0.41	0.14	0.29	0.21	1.93
7	0.76	0.25	0.33	0.23	0.29	0.24	2.10
8	0.84	0.43	0.26	0.27	0.31	0.21	2.32
9	0.78	0.56	0.18	0.18	0.65	0.69	3.04
10	1.00	1.17	0.30	0.34	0.86	0.67	4.34
11	1.43	2.35	0.27	0.36	0.79	0.78	5.98

mM/ml.

This chromatographic method described above was applied to analysis of the contents of volatile fatty acids in the culture medium and in the perienteric fluid of *Ascaris*. The results obtained are illustrated in Table III.

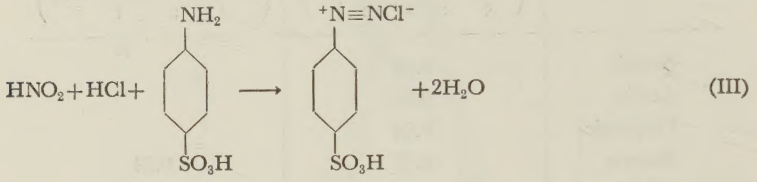
DISCUSSION

Separation procedures used in this paper were similar to that described by Fink *et al.* (*4*).

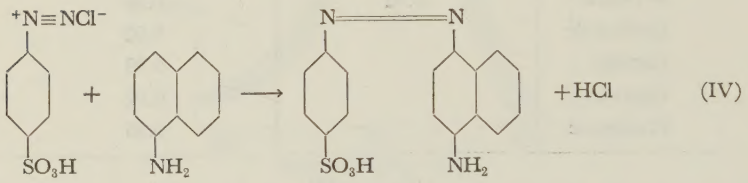
However, this method based on the measurement of optical density of hydroxamate-ferric ion complex was not suitable for analyzing the micro-quantity of fatty acids, which were found in *Ascaris* metabolites. On the other hand, the color intensity of *p*-benzenesulfonyl-azo-naphthylamine obtained by the following reaction formulas was ten times higher than that of hydroxamate-ferric ion complex.

$$RCO(NHOH)+H_2O+HCl \longrightarrow RCOOH+NH_2OH\cdot HCl \qquad (I)$$

$$NH_2OH+2I+H_2O \longrightarrow HNO_2+4HI \qquad (II)$$



(III)



(IV)

According to Feigl (*5*), this reaction proceeds with the splitting of NHOH-group of hydroxamate as $NH_2OH\cdot HCl$ (I). The hydroxylamine is then oxidized by iodine to nitrous acid (II), which diazotize sulfanilic acid (III), and this *p*-diazoniumbenzenesulfonic acid undergoes coupling with α-naphthylamine to produce the red azo dyestuff, *p*-benzenesulfonyl-azo-α-naphthylamine (IV) in acid solution.

Aldoximes and ketoximes also split off NHOH group, and free hydroxylamine, whicn was used in preparation of hydroxamates described above, interferes with this quantitative method, but these disadvantages were eliminated by the use of paper chromatography.

In the case of hydroxamate-ferric complex, the molecular coefficients were relatively varied; the intensity of lower acid was larger than that of higher.

In this method, however, all of the acyl-hydroxamic acids have the same color intensity, for they always split off one molecule of hydroxylamine and the splitting potency is the same for all of hydroxamates.

The outlines of lipid metabolism of *Ascaris* were reported by Flury (*8*) and he established the presence of volatile and non-volatile fatty acids. Bueding (*3*) fractionated the fatty acids excreted *in vitro* by column chromatography. They consisted of acetic acid (20–25 per cent), propionic acid (10 per cent), butyric acids (2–5 per cent), valeric acids (40 per cent), and caproic acids (20–30 per cent). Valeric (C_5) acids consisted of tiglic acid (30 per cent), α-methylbutyric acid (50 per cent) and *n*-valeric acid (20 per cent). Moyle *et al.* (*3*), however, identified α-methylbutyric acid as the

chief component of C_5-acid fraction in the perienteric fluid, and no unsaturated acid such as tiglic acid was found.

TABLE IV

Contents of Lower Fatty Acids in Culture Medium of Ascaris

Days	C_1	C_2	C_3	C_4	C_5	C_6	Total
1	170	130	20	27	150	64	561
2	165	100	13	24	140	35	477
3	170	32	11	6	12′	21	360
4	41	20	15	7	121	62	266
5	62	70	22	14	131	86	385
6	100	20	13	17	147	26	323
7	66	31	18	34	98	27	274
8	20	27	16	40	88	30	221
9	42	27	15	13	71	36	204
10	131	35	68	29	93	35	391
11	96	60	56	11	100	41	364
12	117	27	27	55	73	35	334
13	126	50	27	14	89	26	332

μM/10g./24 hrs.

Although the isomers of C_5 acids were not separated on paper chromatogram, the chief component was formic acid in both the excretory medium and perienteric fluid in this experiment and the contents of each fatty acid changed day by day when *Ascaris* was cultured *in vitro*.

However, the metabolism of fatty acids and the origin of such amount of fatty acids produced by *Ascaris* have not yet been clarified. Recently, Saz *et al.* (*9*) suggested pyruvic acid to be a source of tiglic acid and α-methylbutyric acid, but these reaction routes have not experimentally been proved.

SUMMARY

1. A new sensitive method for determination of lower fatty acids based on Feigl's principles is reported. The method consists of the following sequences:

(a) The separation of acyl-hydroxamic acids by paper chromatography.
(b) Oxidation of hydroxylamine by iodine to nitrous acid.
(c) Coupling of diazotized sulfanilic acid with α-naphthylamine to form stable red azo dyestuff.

2. The method has a lower limit of 0.5 μg. per ml. of fatty acid in solution.

3. This new method was applied to analyze volatile fatty acids in *Ascaris*.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

THE EFFECT OF PUROMYCIN ON RIBONUCLEIC ACID AND PROTEIN SYNTHESIS

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(Received for publication, January 14, 1960)

Puromycin, an antibiotic isolated from *Streptomyces alboniger*, has been found to possess activity against various microorganisms (*1*) and tumors (*2*). From the structure of puromycin, presented in Fig. 1, it has been thought that the antibiotic activity of this substance might be due to inhibition of the synthesis of purines or nucleic acids, and some supporting evidences have been accumulated. For example, Hewitt *et al.* (*3*) reported that, with *Trypanosoma equiperdum* infections in mice, the inhibitory activity of puromycin was reversed partially by a number of purines. Bortle and Oleson (*4*) found that guanylic acid was a competitive antagonist for puromycin in *Tetrahymena pyriformes*. Hutchings (*5*) also made a similar observation with *Lactobacillus plantarum*.

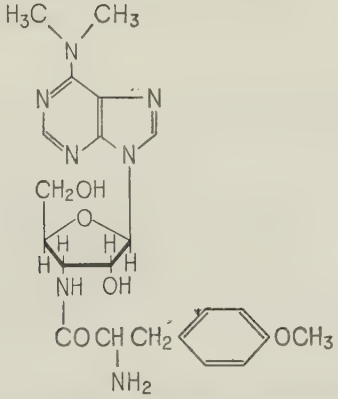

FIG. 1. Chemical structure of puromycin.

In recent years, it has become increasingly familiar that some antibiotics, such as chloramphenicol, are powerful tools for studies on the relationship between nucleic acid and protein synthesis. Thus, Gale and Folkes (*6*), and Wisseman *et al.* (*7*) found that chloramphenicol inhibits protein but not nucleic acid synthesis in sensitive bacteria. Neidthardt and Gros

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(*8*), and Hahn *et al.* (*9*) observed that the RNA* which was accumulated in the presence of chloramphenicol was extremely unstable *in vivo* and broken down upon the removal of the antibiotic. This result attracted much attention bacause of a marked difference from the stability of RNA formed under normal conditions (*10*, *11*).

The experiments which will be reported here were undertaken to study the effect of puromycin on nucleic acid and protein biosynthesis in *Pseudomonas fluorescens*, strain A_{3-12}. It was found that, like chloramphenicol, puromycin abolishes protein but not RNA synthesis in opposition to the hypothesis previously mentioned. However, the stability of the RNA accumulated with puromycin is in sharp contrast to that with chloramphenicol.

EXPERIMENTAL

Bacterial Cultures—The strain used was *Pseudomonas fluorescens*, strain A_{3-12}, obtained through the courtesy of Dr. R. Y. Stanier of the University of California, U. S. A. Exponential growth phase cultures were prepared by overnight growth on a basal medium (Na_2HPO_4, 3 g.; KH_2PO_4, 2 g.; $(NH_4)_2SO_4$, 2.5 g.; NaCl, 1 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $CaCl_2 \cdot 2H_2O$, 10 mg. and $FeSO_4 \cdot 7H_2O$, 3 mg. per liter; adjusted to pH 7.2 with NaOH), containing 0.5 per cent sodium citrate (medium A). They were transferred to a fresh medium and regrown.

When a medium low in magnesium was desired, the magnesium concentration of medium A was reduced to 0.02 g. per liter (medium B).

The medium for isotopic experiments using inorganic P^{32} was: sodium citrate, 5 g.; $(NH_4)_2SO_4$, 2.5 g.; NaCl, 1 g.; KH_2PO_4, 50 mg.; $MgSO_4 \cdot 7H_2O$, 20 mg.; $CaCl_2 \cdot 2H_2O$, 10 mg.; $FeSO_4 \cdot 7H_2O$, 3 mg. and tris(hydroxymethyl)aminomethane, 6 g. per liter; adjusted to pH 7.2 with HCl (medium C). All incubations were carried out at 25° with shaking.

Analytical Procedures—The growth of the cultures was followed turbidimetrically with a Coleman junior-type spectrophotometer at 550 mμ.

RNA and DNA were measured by colorimetric analyses. Aliquots of the cultures were centrifuged, washed twice with 5 per cent TCA in the cold, and then heated with 5 per cent TCA at 100° for 30 minutes. After centrifugation, the precipitate was suspended in 5 per cent TCA and recentrifuged. The combined supernatants were analyzed for ribose by the orcinol method (*12*) and for deoxyribose by the diphenylamine method (*13*). Protein was determined by the biuret reaction.**

In the isotopic experiments, RNA-P^{32} was separated according to the Schmidt Thanhauser-Schneider method (*14*) and the radioactivity measured in an end-window Geiger counter.

Puromycin Treatment of Cells—Exponential growth phase cultures in medium A were harvested by centrifugation, washed once with distilled water and suspended in medium B at an optical density of 0.200. At 0 time sufficient puromycin was added to the suspension to give a final concentration of 50 μg. per ml. The suspensions were reincubated with shaking for 2 to 3 hours at 25°. In isotopic experiments, medium B was replaced by medium C.

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* The abbreviations used are: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; TCA, trichloroacetic acid.

** Pardee, A. B., unpublished

Materials—Puromycin was kindly supplied by Dr. J. Kawamata of the Institute for Microbial Diseases, Osaka University, and by Dr. B. L. Hutchings of the Research Division, American Cyanamid Company, U. S. A. Chloramphenicol was obtained from the Sankyo Pharmaceutical Company, Japan.

RESULTS AND DISCUSSION

Relationship between Puromycin Activity and the Magnesium Concentration—In preliminary experiments, results suggested that there was a competitive relation between magnesium and puromycin. This was examined and the results are shown in Fig. 2.

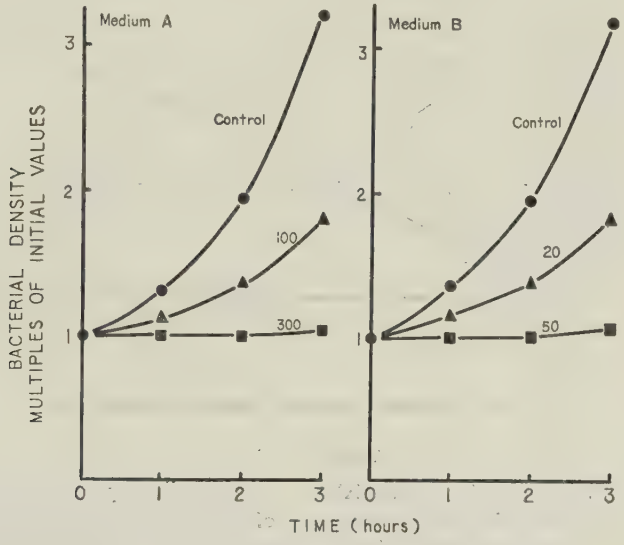

FIG. 2. Growth curves of *Pseudomonas fluorescens*, strain A_{3-12}, with various concentrations of puromycin and magnesium. Bacteria were incubated in medium A (magnesium sulfate, 500 μg. per ml.) or in medium B (magnesium sulfate, 20 μg. per ml.) in the presence of the amounts of puromycin indicated (μg. per ml.). Temperature 25°. Bacterial densities were measured as described in the text and plotted as multiples of the initial values.

It is clear that the higher the concentration of magnesium, the more puromycin is required to inhibit cell growth. For example, 300 μg. per ml. of puromycin is necessary to prevent multiplication in the presence of 500 μg. per ml. of magnesium sulfate, whereas only 50 μg. per ml. of puromycin is required when the magnesium is reduced to 20 μg. per ml.

Therefore in subsequent experiments medium B rather than medium A was used. The exact nature of the effect is still not clear.

Accumulation of Nucleic Acids during Puromycin Treatment—From a consideration of the structure of puromycin, it was first thought that it might exhibit

an antibiotic activity by blocking nucleic acid synthesis.

Exponential phase cells of *Pseudomonas fluorescens*, strain A_{3-12}, grown on medium A, were transferred to medium B and incubated at 25° in the presence of puromycin. During the incubation, aliquots were removed and their protein, RNA and DNA contents measured. The results are given in Fig. 3. Contrary to expectations, puromycin did not inhibit RNA synthesis, whereas it completely abolished protein formation. DNA also doubled during the incubation and then remained constant.

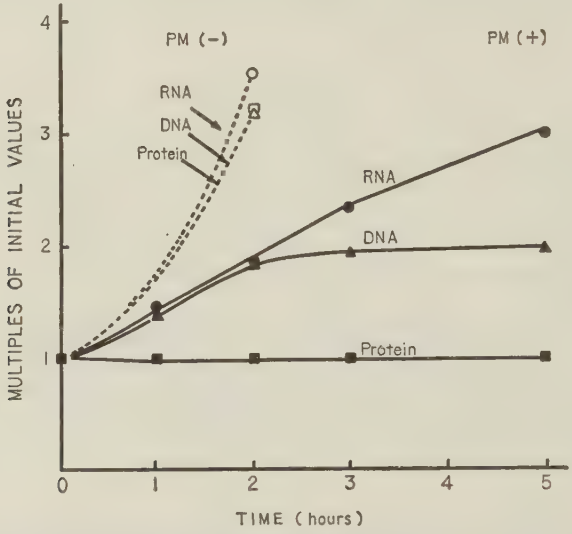

Fig. 3. Changes in protein, RNA and DNA during puromycin treatment. Exponential growth phase cells of *Pseudomonas fluorescens*, strain A_{3-12}, were incubated at 25° in medium B in the presence (50 μg. per ml.) or absence of puromycin (PM). At intervals aliquots were taken from the culture and analyzed for protein, RNA and DNA as described in the text. The results are plotted as multiples of the initial values.

The accumulation of RNA was further confirmed by another experiment in which P^{32}-orthophosphate was used as a marker. As shown in Fig. 4, incorporation of P^{32}-orthophosphate into RNA was not affected significantly by puromycin. In a further experiment, incorporation of methionine-S^{35} into protein was found to be completely suppressed under the same conditions.

Stability of the RNA Formed in the Presence of Puromycin—Two types of abnormal accumulation of RNA in microorganisms have been reported. One is caused by chloramphenicol treatment and the RNA accumulated is very unstable and rapidly broken down *in vivo* upon removal of the antibiotic. The degradation of the RNA occurs both in growing and in resting cells. The other type is the one reported by Borek and Ryan (*15*). When a methionine-requiring strain of *E. coli* is cultivated in a methionine-free

medium, RNA is accumulated without concomitant synthesis of protein. A characteristic of this type is that the RNA formed is stable, thus being in sharp cantrast to the former.

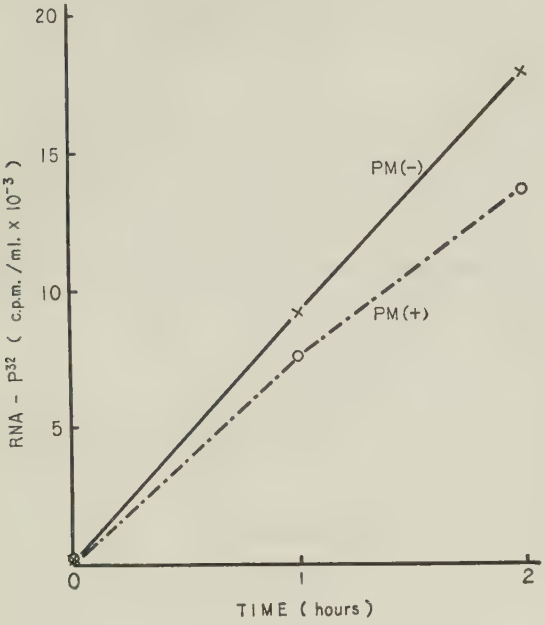

FIG. 4. Incorporation of P^{32}-phosphate into cellular RNA during puromycin treatment. Bacteria were incubated in medium C for 2 hours at 25° with P^{32}-phosphate (22,700 c.p.m. per ml.) and with or without puromycin (50 μg. per ml.). The radioactivity of the RNA was measured as described in the text.

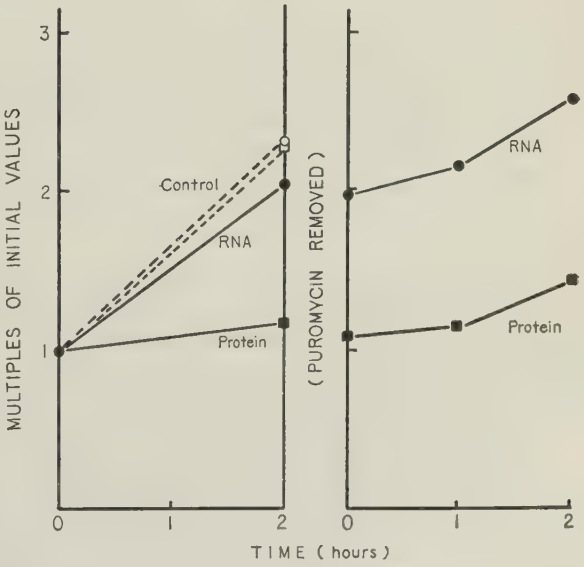

FIG. 5. Changes in protein and RNA of *Pseudomonas fluorescens*, strain A_{3-12}, exposed to, or recovering from the action of puromycin. Bacteria were incubated at 25° for 2 hours in medium B containing 40 μg. per ml. of puromycin. They were then centrifuged, washed with water and again incubated in medium A. At intervals, aliquots were taken from the culture for analysis of protein and RNA as described in the text.

The results are plotted as multiples of the initial values.

It is of interest to know to which category the RNA accumulated in our system belongs. Therefore, bacteria which had accumulated RNA with

puromycin, were reincubated in a puromycin-free medium after washing out the antibiotic.

Fig. 5 shows a typical result of such experiments. After about one hour of reincubation the bacteria began to grow again. The amount of RNA did not show any decrease during this period and started to increase in parallel with cell growth. This shows that the RNA formed in the presence of puromycin is stable in growing cells.

It seems likely that the same would be true in resting cells. Experiments were conducted with the aid of P^{32}-orthophosphate. Cells which had accumulated labeled RNA in the presence of puromycin were washed, suspended in phosphate buffer and reincubated. Aliquots were removed every hour and the radioactivity of the RNA fractions measured. As shown in Table I, the radioactivity of the RNA remained constant during the experiment. The result shows that RNA formed in the presence of puromycin is stable in resting cells.

TABLE I

Stability of the RNA Formed in the Presence of Puromycin

Time after the removal of puromycin	RNA-P^{32}
minutes	*c.p.m. per ml.*
0	7250
30	6970
60	7010
90	7070

Cells which had been incubated at 25° for 3 hours in medium C with puromycin (50 μg. per ml.,) and P^{32}-phosphate (17,000 c.p.m. per ml.) were transferred to 0.05 *M* phosphate buffer, pH 7.2, and again incubated for 90 minutes.

The radioactivity of cellular RNA was measured as described in the text.

An attempt was made to find differences in the physicochemical properties of the RNA formed in the presence and absence of puromycin. For this study, the RNA samples were extracted from the cells and purified by the phenol method (*16*). The preparations were then analyzed physicochemically. However, no significant difference in electrophoretic, ultracentrifugal or ECTEOLA-cellulose chromatographic patterns was found out.

Instability of the RNA Formed in the Presence of Chloramphenicol—From the preceding experiments, it is obvious that the RNA accumulated in the presence of puromycin differs in stability from that formed by chloramphenicol treatment in *E. coli*. However, the question arises of whether this difference in stability is simply due to a difference in the type of organism used. Therefore *Pseudomonas fluorescence*, strain A_{3-12}, was incubated with

TABLE II

Degradation of P^{32}-Labeled RNA Accumulated during Chloramphenicol Treatment

Time after the removal of chloramphenicol	Bacterial density	RNA-P^{32}
minutes		*c.p.m. per ml.*
0	0.140	2580
30	0.140	1240
60	0.150	1370
90	0.250	1040
120	0.350	1390

Cells of *Pseudomonas fluorescens*, strain A_{3-12}, were incubated for 2 hours in medium C containing chloramphenicol (50 μg. per ml.) and P^{32}-phosphate (5,500 c.p.m. per ml.). After incubation cells were centrifuged, washed and reincubated in medium B.

The bacterial density and the radioactivity of RNA were measured as described in the text.

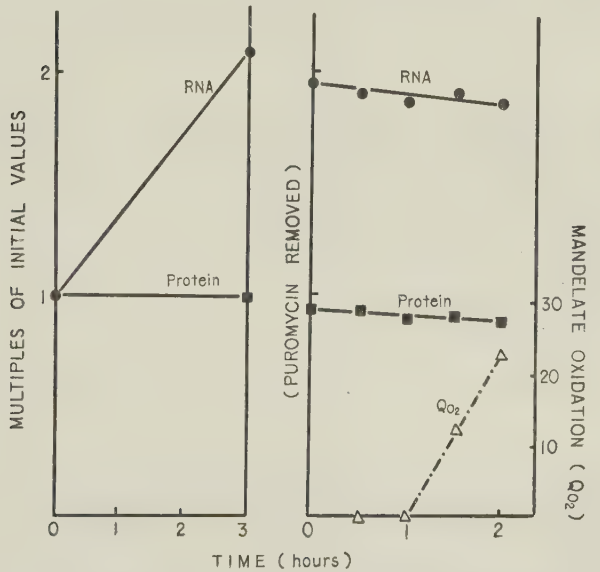

FIG. 6. Formation of adaptive enzymes by puromycin-treated *Pseudomonas fluorescens*, strain A_{3-12}. Cells were incubated for 3 hours in medium B with puromycin (50 μg. per ml.). They were centrifuged, washed and reincubated in 0.04 *M* phosphate buffer, pH 7.2, containing 10^{-3} *M* mandelate. Temperature 25°. Aliquots of the bacterial suspension were assayed for mandelate oxidizing enzymes in a Warburg respirometer. The assay system was as follows: 1 ml. of washed bacterial suspension in 0.04 *M* phosphate buffer, pH 7.2, optical density, 0.2; sodium mandelate, 2 μmoles; chloramphenicol, 40 μg. Volume 2 ml. Temperature 25°. Activities expressed as Q_{O_2}. Analytical results are plotted as multiples of the initial values.

P^{32}-orthophosphate in the presence of chloramphenicol and allowed to accumulate labeled RNA. After washing, the cells were transferred to chloramphenicol-free medium (medium B) and incubated with shaking at 25°. During the incubation, aliquots were removed and the radioactivities in the RNA fractions measured. As can be seen from Table II, the cells began to grow again after a certain lag phase. However the P^{32}-content of the RNA fraction dropped sharply by almost 50 per cent during the latent period. The result shows that RNA formed by this organism, as by *E. coli*, in the presence of chloramphenicol is labile upon removal of the antibiotic.

Effect of Puromycin Treatment on Formation of Induced Enzymes—It is of interest to see what effects the abnormal accumulation of RNA exerts on cell metabolism. Therefore adaptation to mandelic acid in treated and untreated cells was compared. Cells of *Pseudomonas fluorescens*, strain A_{3-12}, which had been preincubated for 3 hours in the presence of puromycin, were washed and suspended in phosphate buffer, and adaptation to mandelate was examined. As can be seen from Fig. 6, the treated cells began to synthesize a mandelate oxidizing enzyme system after a latent period of about one hour, and there was no difference in behaviour from untreated cells. It should be mentioned that the RNA content of the treated cells was maintained at a high level during formation of induced enzymes.

SUMMARY

1. Puromycin completely inhibits protein synthesis but not RNA and DNA synthesis in *Pseudomonas fluorescens*, strain A_{3-12}.

2. The RNA accumulated in the presence of puromycin is quite stable both in growing and in resting cells on removal of the antibiotic, whereas the one accumulated by the same bacteria in the presence of chloramphenicol is very unstable.

3. The RNA accumulated does not exert any siginificant effect on induced enzyme formation.

The authors wish to thank Prof. M. Suda for his consultation and encouragement.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

TURNIP PEROXIDASE

IV. THE EFFECT OF pH AND TEMPERATURE UPON THE RATE OF REACTION

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(Received for publication, February 1, 1960)

The physicochemical properties and the reaction mechanisms of the three kinds of turnip peroxidases, *i.e.* TP-A_1[1], TP-A_2[1], and TP-D[1], were reported previously (*1–3*).

In the present paper are described the results of the experiments dealing with the effect of the hydrogen ion concentration and temperature upon the rate of reaction catalyzed by the enzymes. This study has a dual purpose. The first is to characterize the nature of the three enzymes, while the second is to ascertain whether or not any amino acid residue essential for the activity of the enzymes exists in the protein moiety.

Little information is available on the essential group of the protein moiety of peroxidases, although the investigations merely aimed at obtaining the value of the apparent optimum pH have been made by many workers (*4*). The effect of pH upon the individual reaction steps of HRP[1] was first investigated by Chance (*5–7*), who found that the dissociation constant of the enzyme-substrate complex I (Complex I) and the rate constant of the reaction of the enzyme-substrate complex II (Complex II) with various hydrogen donor molecules are very little affected by the hydrogen ion concentration in the pH range 3.6~8.8 and 3.5~6.7, respectively. Accordingly, it was suggested by him that hydrogen and hydroxyl ions are not involved in these reactions. However, Tonomura (*8*) inferred, on the basis of the results of his kinetic studies of Japanese-radish peroxidase, that a group having the pK value of 7.4 in the enzyme protein is essential for the enzymatic reaction.

The present experiments that were made using turnip peroxidases clearly show that the rate of reaction of Complex II with hydrogen donor molecule is remarkably affected by pH in the alkali-region. The analysis of the data suggests that an α-ammonium group of the enzyme is essential for the reaction.

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1) The following abbreviations are used: TP-A_1, TP-A_2 and TP-D, turnip peroxidases A_1, A_2 and D; HRP, horseradish peroxidase II.

MATERIALS AND METHODS

Materials—TP-A_1, TP-A_2 and TP-D used in the present work were prepared as described previously (*1*). Buffer solutions used were acetate in the pH range 3.7～5.9, phosphate in the pH range 5.8～8.3, and tris(hydroxylmethyl)aminomethane-HCl in the pH range 8.4～9.4. The concentration of acetate or phosphate in the reaction mixture was 32 mM, and that of tris(hydroxylmethyl)aminomethane was 16 mM. The pH of the reaction mixture was measured by means of a Beckman model G glass electrode pH meter.

Methods—The rate of over-all reaction (v_0) was measured by the method[2] described previously (*2*), but the values of the rate constants were determined by means of the following simplified procedure[3] instead of the more strict method described there.

The rate of over-all reaction depends on the respective concentrations of hydrogen peroxide and hydrogen donor in the following way (*2*):

$$\frac{e}{v_0} = c_1 + \frac{1}{\tilde{k}_1 x_0} + \frac{1}{\tilde{k}_d a_0} \qquad \text{Eq. (1),}$$

where e, x_0 and a_0 are the initial concentrations of enzyme, hydrogen peroxide, and hydrogen donor, respectively, and c_1 is a constant independent of x_0 and a_0, and $\tilde{k}_1$[4] and $\tilde{k}_d$ [4] [5] are the rate constant of the reaction of formation of Complex I and that of the reaction of Complex II with hydrogen donor, respectively. Accordingly, if the conditions of the reaction are chosen so that $\tilde{k}_d a_0 \gg \tilde{k}_1 x_0$, and if c_1 is negligibly small in comparison with $1/(\tilde{k}_1 x_0)$[6] we have

$$\frac{e}{v_0} = \frac{1}{\tilde{k}_1 x_0} \qquad \text{Eq. (2),}$$

hence,

$$\tilde{k}_1 = \frac{v_0}{e x_0} \qquad \text{Eq. (3).}$$

On the other hand, if the conditions of the reaction are adjusted so that $\tilde{k}_1 x_0 \gg \tilde{k}_d a_0$, and if c_1 is negligibly small in comparison with $1/(\tilde{k}_d a_0)$[6] we have

2) The millimolar extinction coefficient at 470 mμ of guaiacol in the oxidized form, ε_{470}, indispensable to the experimentation by the method, was obtained by the procedure described previously (*2*) in the pH range studied. It was found to be somewhat dependent on pH as indicated in the following table:

pH	4.0	4.5	5.0	5.5	6.0	6.5	7.0	8.0	9.0
ε_{470} (cm.$^{-1}$ mM^{-1})	6.95	6.87	6.74	6.48	6.11	5.77	5.57	5.56	5.57

3) This procedure is essentially the same as that described by Chance and Maehly (*9*).

4) The rate constant with a wave sign means the apparent rate constant and that without a wave sign the intrinsic rate constant.

5) The notation "$\tilde{k}_d$" is used here for convenience expressing the reciprocal of c_3 in Eq. (2) of the second paper of this series (*2*), since c_3 is solely concernig with the rate constants of the reactions of Complex I and II with hydrogen donor. It is easily found that $\tilde{k}_d$ is approximately equal to $\tilde{k}_4$ or $\tilde{k}_9/\tilde{K}_M$, assuming Mechanism A or B of the second paper, respectively.

6) It has been confirmed that this condition is fulfilled in almost all experiments of the present work, taking into account the values of c_1 given previously (*2*).

$$\tilde{k}_d = \frac{v_0}{e\, a_0} \qquad \text{Eq. (4).}$$

RESULTS

I. Effect of Hydrogen Ion Concentration

Effect of pH upon $\tilde{k}_d$ of TP-D—The value of $\tilde{k}_d$ of TP-D for guaiacol was obtained in the pH region 3.9~9.4 and at three different temperatures (4°, 20° and 32°), the results being summarized in Fig. 1, where the ratio of the observed value of $\tilde{k}_d$ at a given pH to the value of k_d^{max} (the maximum value of $\tilde{k}_d$ throughout the pH range studied) was plotted against pH. As may be seen in this figure, the $\tilde{k}_d/k_d^{max}$ -pH curves represent approximately "sigmoid curves of the first order" in the region of pH above 6. In other words, these curves may be represented by the equation:

$$\frac{\tilde{k}_d}{k_d^{max}} = \frac{[H^+]}{\phi + [H^+]} \qquad \text{Eq. (5),}$$

where ϕ is a constant corresponding to the hydrogen ion concentration causing 50 per cent inhibition. The values of ϕ are estimated from Fig. 1 to be $10^{-8.2}$ at 4°, $10^{-7.8}$ at 20° and $10^{-7.5}$ at 32°. The value of $\tilde{k}_d$ in the more acidic region than pH 5 shows tendency to decrease as shown in this figure. It is, however, difficult to analyze the effect of pH, since $\tilde{k}_1$ decreases in the cor-

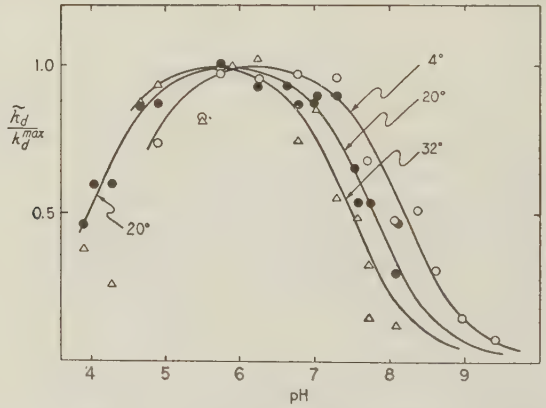


FIG. 1. Effect of pH and temperature upon $\tilde{k}_d$ of TP-D.

The value of $\tilde{k}_d$ of TP-D was obtained at 4°, 20° and 32° using guaiacol as hydrogen donor in the pH range 3.9~9.4 by the method described in the text, and the ratio of $\tilde{k}_d$ to k_d^{max} was plotted against pH. The curves in the alkaline side were drawn theoretically by the use of Eq. (5) assuming the values of ϕ to be $10^{-8.2}$ at 4°, $10^{-7.8}$ at 20°, and $10^{-7.5}$ at 32°. The reaction mixture contains hydrogen peroxide, $2.14\times10^{-4}M$; guaiacol, $1.74\times10^{-5}M$; the enzyme, 0.58×10^{-9} to $2.0\times10^{-9}M$. The condition of $\tilde{k}_1 x_0 \gg \tilde{k}_d a_0$ is nearly fulfilled, since $\tilde{k}_1 x_0$ and $\tilde{k}_d a_0$ are estimated to be 900 sec.$^{-1}$ and 90 sec.$^{-1}$ at pH 7.0, respectively.

responding pH region as described below and the splitting of hematin from protein moiety may occur below pH 4 as in the case of HRP (*10*, *11*).

Effect of pH on the e/v_0-$1/a_0$ relationship—In order to check whether or not the hydroxyl ion is a competitive inhibitor for the reaction of Complex II with the hydrogen donor molecule, the e/v_0-$1/a_0$ relationship was examined in the medium of different pH values (pH 6.15, 7.02 and 7.70), the results being illustrated in Fig. 2. As may be seen in this figure, these lines meet

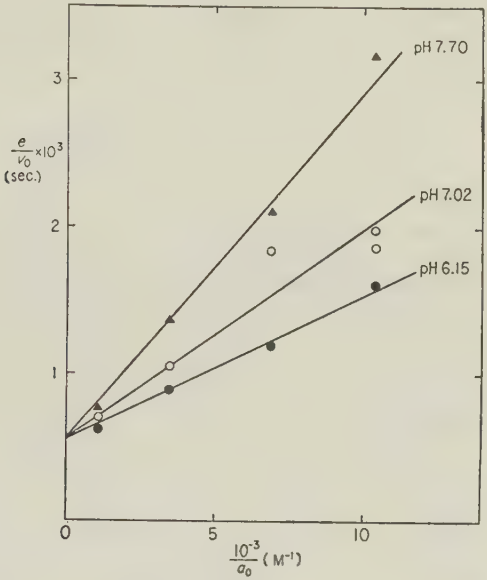


FIG. 2. Relationship between e/v_0 and $1/a_0$ at different concentrations of hydrogen ion.

The value of v_0 was obtained at 20° and pH 6.15, 7.02 or 7.70 by the method described in the text using TP-D, and the value of e/v_0 was plotted against $1/a_0$ at each pH. The reaction mixture contains hydrogen peroxide, $2.2\times10^{-4}M$; guaiacol, 8.8×10^{-5} to $9.8\times10^{-4}M$; the enzyme, $5.1\times10^{-10}M$.

at a point on the ordinate, suggesting that the hydroxyl ion competes with the hydrogen donor in the reaction between Complex II and the hydrogen donor.

Effect of pH upon $\tilde{k}_d$ of TP-A_1 and of TP-A_2—The values of $\tilde{k}_d$ of TP-A_1 and of TP-A_2 were obtained at various pH's (4.1~8.2) and at a constant temperature (20°), and the ratio of $\tilde{k}_d$ at a given pH to k_d^{max} was plotted against pH as shown in Fig. 3. As evident from this figure, the curves also represent in the alkaline side "sigmoid curves of the first order" that are expressed by Eq. (5). The values of ϕ are estimated to be $10^{-7.8}$ for TP-A_1 and $10^{-6.9}$ for TP-A_2.

Effect of pH upon $\tilde{k}_1$ of TP-D—The value of $\tilde{k}_1$ of TP-D was determined at various pH's (3.8~9.2) and at a constant temperature (20°), and the ratio

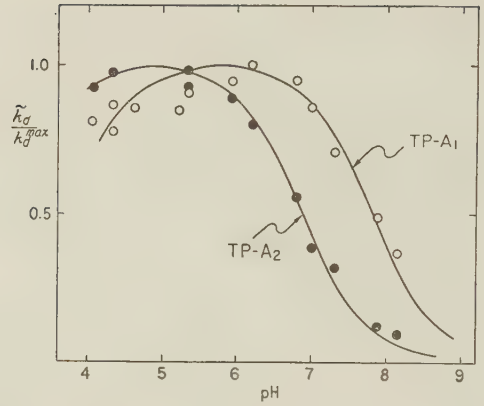


FIG. 3. Effect of pH upon $\tilde{k}_d$ of TP-A_1 and of TP-A_2.

The value of $\tilde{k}_d$ was obtained at 20° in the pH range 4.1~8.2, and the ratio of $\tilde{k}_d$ to k_d^{max} was plotted against pH. The reaction mixture contains hydrogen peroxide, $1.53\times10^{-4}M$; guaiacol, $3.33\times10^{-3}M$; the enzyme, $2.50\times10^{-8}M$. The condition of $\tilde{k}_1x_0 \gg \tilde{k}_da_0$ is fulfilled since the values of $\tilde{k}_1x_0$ and $\tilde{k}_da_0$ are estimated to be 1500 sec.$^{-1}$ and 12 sec.$^{-1}$ in the case of TP-A_1, 200 sec.$^{-1}$ and 17 sec.$^{-1}$ in the case of TP-A, at pH 7.0, respectively.

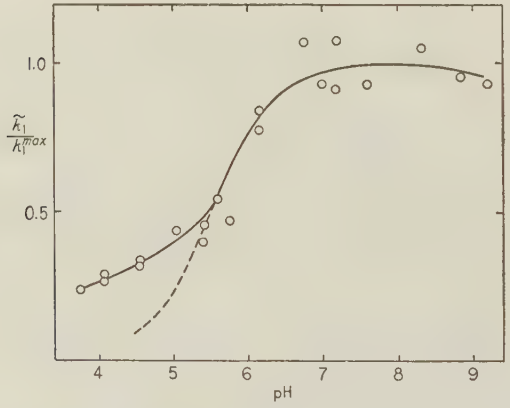


FIG. 4. Effect of pH upon $\tilde{k}_1$ of TP-D.

The value of $\tilde{k}_1$ of TP-D was obtained at 20° by the method described in the test using guaiacol as hydrogen donor, and the ratio of $\tilde{k}_1$ to k_1^{max} was plotted against pH. The dotted line shows a sigmoid curve of the first order having the pϕ value of 5.5. The reaction mixture contains hydrogen peroxide, $3.6\times10^{-5}M$; guaiacol, $1.0\times10^{-3}M$; the enzyme, $1.4\times10^{-9}M$. The condition of $\tilde{k}_1x_0 \ll \tilde{k}_da_0$ is fulfilled since the values of $\tilde{k}_1x_0$ and $\tilde{k}_da_0$ are estimated to be 200 sec.$^{-1}$ and 5900 sec.$^{-1}$, at pH 7.0, respectively.

of $\tilde{k}_1/k_1^{max}$ was plotted against pH, as shown in Fig. 4. The value of $\tilde{k}_1$ was nearly constant in the pH range 6~9, but it tended to decrease below pH

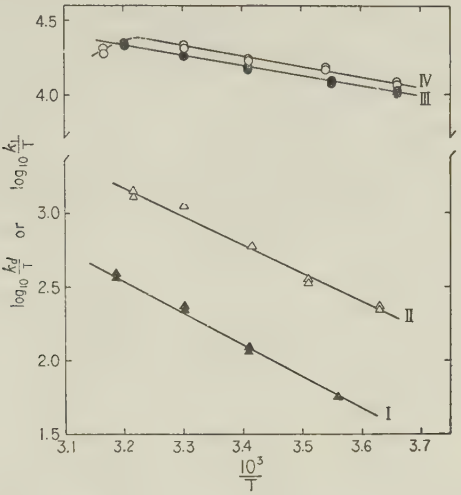


FIG. 5. Effect of temperature upon the rate constants k_1 and k_d.

The ratio of the rate constant to absolute temperature is plotted against the reciprocal of absolute temperature. The conditions of the reaction mixture are as follows:

I: k_d of TP-A_1; pH 5.62, $x_0=1.53\times10^{-4}M$, $a_0=3.43\times10^{-4}M$.
II: k_d of TP-A_2; pH 4.65, $x_0=7.64\times10^{-4}M$, $a_0=2.74\times10^{-4}M$.
III: k_1 of TP-D; pH 7.30, $x_0=3.64\times10^{-5}M$, $a_0=1.15\times10^{-3}M$.
IV: k_d of TP-D; pH 5.62, $x_0=1.53\times10^{-4}M$, $a_0=3.43\times10^{-4}M$.

TABLE I

The Heat of Activation and the Entropy of Activation of the Rate Constants k_1 and k_d

These values were calculated using the data of Fig. 6. The values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ concerning k_d should be regarded essentially as the resultant of the values of the individual reaction steps (see footnote 5).

Peroxidase	TP-A_1	TP-A_2	TP-D		HRP[a]	
Rate constant	k_d	k_d	k_1	k_d	k_1	k_d
pH of reaction mixture	5.62	4.65	7.30	6.47		
$\Delta H^{\neq}$ (kcal. M^{-1})	10	8.4	3.0	3.4	2.1	6.0
$\Delta S^{\neq}$(e.u.)	−4.4	−4.6	−17	−17	−20	−13

a) These values were calculated from the data of Chance (*13*) by the present author. In this case, k_d can be regarded as k_4.

6 although the $\tilde{k}_1/k_1^{max}$ -pH curve did not represent perfectly "a sigmoid curve of the first order."

II. Effect of Temperature upon Rate Constant and ϕ

Effect of Temperature upon k_1 of TP-D and k_d of TP-A_1, TP-A_2 and TP-D—The experiments under various temperatures (0.1°–40°) were carried out at the constant pH, at which the maximum value of the rate constant was attained. The results obtained are illustrated in Fig. 5, where the logarithms of the ratio of the rate constant to absolute temperature are plotted against the reciprocal of absolute temperature. As evident from this figure, there exists in each case a linear relationship, from which the heat of activation (ΔH^*) and the entropy of activation (ΔS^*) were evaluated (*cf.* (*12*)) and listed in Table I.

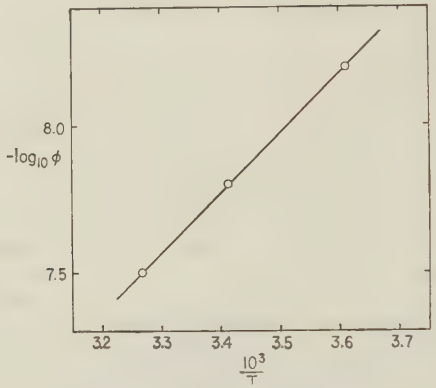


Fig. 6. Relationship between ϕ and absolute temperature.

The values of ϕ, the hydrogen ion concentration causing 50 per cent inhibition, were obtained from Fig. 1.

Effect of temperature upon ϕ of TP-D—When the logarithms of the values of ϕ obtained from the data of Fig. 1 were plotted against the reciprocal of absolute temperature, a straight line was obtained as shown in Fig. 6. The implication of this fact will be discussed below.

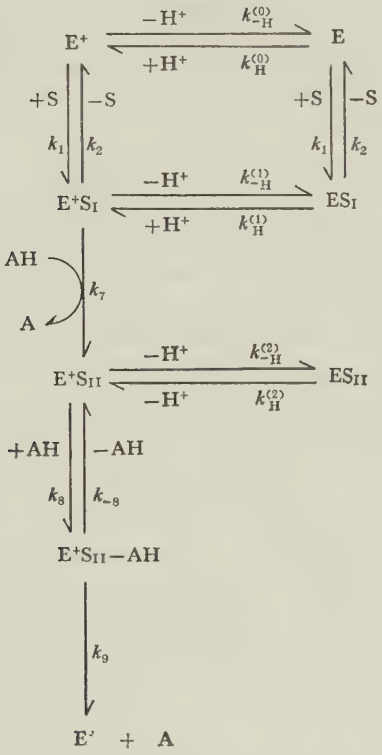
DISCUSSION

The results illustrated in Fig. 1 clearly show that $\tilde{k}_d$ of TP-D is dependent on the hydrogen ion concentration in the pH range 6~9, where the values of $\tilde{k}_1$ remain nearly constant. Such pH dependence of $\tilde{k}_d$ suggests[7] that there exists an ionizable group of the protein moiety, which is essential for the

7) There is another possibility that the hydroxyl ion may combine with the prosthetic group of Complex II, and diminish the reactivity of Complex II with the hydrogen donor. However, the possibility may be disregarded since it has been found by the present author that the absorption spectrum of Complex II of TP-D does not change in the pH range 7~9 as in the case of HRP (*14*).

reaction of Complex II with guaiacol, since the pK value of guaiacol molecule is 9.93 at 25°, lying outside the pH region studied. Moreover, the experimental result illustrated in Fig. 2 suggests that, if we assume Mechanism B described previously (*2*), the hydroxyl ion competes with the hydrogen donor molecule for the combination with Complex II, thus causing the decrease of $\tilde{k}_d$ (cf. (*2*, *15*)). Accordingly, it is reasonable to propose the following scheme[8]:

Scheme I



Here E^+ and E represent the free enzyme, E^+S_I and ES_I Complex I, E^+S_{II} and ES_{II} Complex II, E^+S-AH the "ternary complex," H^+ a proton, AH and A a hydrogen donor molecule in the reduced and oxidized form, respectively, S a hydrogen peroxide molecule. The plus sign on the shoulder of E shows that the essential group of the protein moiety contains a proton, thus being the active form. The notation k stands for the rate constant of the respective reaction. In this scheme, it is assumed that AH can combine only with

8) 9) In these schemes, the consideration on the acidic branch of the activity-pH curves is omitted, and the rate constant of the reaction of Complex I with the hydrogen donor is assumed to be sufficiently great compared to that of Complex II with the hydrogen donor. The notations, E^+, E^+S_I, and E^+S_{II} in these schemes correspond to E, ES_I, and ES_{II} in the previous report (*2*), respectively.

E^+S_{II}, but not with ES_{II}, to break down into the reaction product and the free enzyme. On the basis of this scheme, the following equation can be deduced:

$$\frac{e}{v_0}=\frac{1}{k_9}+\frac{1}{k_1 x_0}+K_M\times\frac{1}{k_9}\times\left(1+\frac{k_{-H}^{(2)}}{k_H^{(2)}}\times\frac{1}{[H^+]}\right)\times\frac{1}{a_0} \qquad \text{Eq. (6).}$$

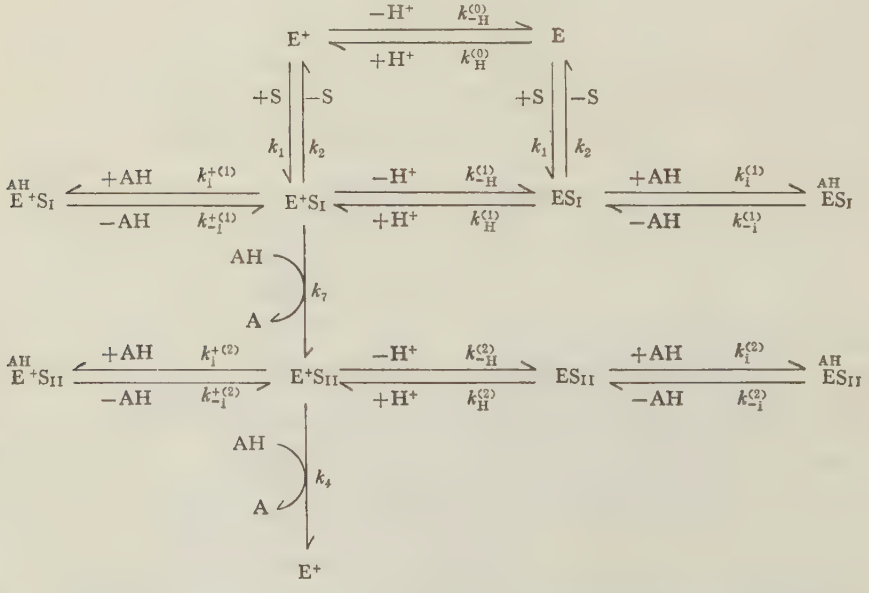
This equation corresponds satisfactorily with the experimental results illustrated in Fig. 2, indicating that the plots of e/v_0 *versus* $1/a_0$ at various concentrations of hydrogen ion give straight lines which meet together at a point on the ordinate. In the conditions of the reaction adjusted so that $\tilde{k}_1 x_0$ is far greater than $\tilde{k}_d a_0$, the following relationship can be easily deduced using Eq. (6):

$$\frac{v_0}{v_0^{max}}=\frac{\tilde{k}_d}{k_d^{max}}=\frac{[H^+]}{\frac{k_H^{(2)}}{k_{-H}^{(2)}}+[H^+]} \qquad \text{Eq. (7).}$$

This equation coincides well with Eq. (5) already established experimentally, ϕ in Eq. (5) being $k_H^{(2)}/k_{-H}^{(2)}$, the ionization constant (K_a).

On the other hand, in the case where the alternative mechanism (Mechanism A) is assumed (*2*), the following scheme[9] must be set up, in order to explain the results illustrated in Fig. 2, assuming that AH can react with E^+S_{II}, but not with ES_{II}, to be oxidized:

SCHEME II



From this scheme is deduced the following equation, which finally leads to the same equation as Eq. (7):

$$\frac{e}{v_0} = c_1 + \frac{1}{k_1 x_0} + \frac{1}{k_4 a_0}\left(1 + \frac{k_{-H}^{(2)}}{k_H^{(2)}} \times \frac{1}{[H^+]}\right) \qquad \text{Eq. (8).}^{10)}$$

However, it may be inferred that this scheme is not probable, since, no matter how the assumption mentioned above is indispensable in this scheme, the assumption seems to be unreasonable from the standpoint of the collision theory on which Mechanism A stands: it is rather difficult to explain how the proton attached to the essential group of the protein moiety of Complex II (see footnote 7) affects the reactivity of the prosthetic group of Complex II for the hydrogen donor molecule. On the contrary, as mentioned already, the scheme based on Mechanism B can explain more easily the experimental results. Thus, it may be inferred here that Mechanism B is the more probable of the two mechanisms, the comparative merits of which could not be determined in the previous paper (*2*).

At any rate, it has been found that ϕ is equal to the ionization constant of the essential group of Complex II, whichever mechanism is assumed. The heat of activation (ΔH) of the essential group of TP-D can, therefore, be evaluated using the results of Fig. 6, according to the well-known equation:

$$\Delta H = -\frac{R}{0.434} \times \frac{d \log_{10} Ka}{d\,(1/T)} \qquad \text{Eq. (9).}$$

where R is gas constant. The value of ΔH was found to be 10 kcal. M^{-1}. Thus, the essential group of TP-D is reasonably supposed to be an α-ammonium group of a certain amino acid in the protein moiety by the reference of the reported values[11] of pK and ΔH of various ionizing group found in proteins (*16*).

In the case of TP-A_1 and TP-A_2, the experiments on the effect of temperature upon ϕ were not performed, and accordingly the values of the heat of activation of the essential groups are unknown. However, it is supposed that the essential group of TP-A_1 is also an α-ammonium group, in view of the fact that the value of ϕ of TP-A_1 agrees with that of TP-D.

10) In this equation, c_1 is essentially dependent on pH, since c_1 is in fact as written as follows,

$$c_1 = \frac{1}{k_7} \times \frac{k_{-i}^{(1)}}{k_i^{(1)}}\left(1 + \frac{k_{-H}^{(1)}}{k_H^{(1)}} \times \frac{1}{[H^+]}\right) + \frac{1}{k_4} \times \frac{k_{-i}^{(2)}}{k_i^{(2)}} \times \left(1 + \frac{k_{-H}^{(2)}}{k_H^{(2)}} \times \frac{1}{[H^+]}\right.$$

assuming $k_i^{+(1)}$, $k_{-i}^{+(1)}$ etc. to be equal to $k_i^{(1)}$, $k_{-i}^{(1)}$ etc., respectively. However, the experimental results illustrated in Fig. 2 shows that the term $\left(c_1 + \frac{1}{k_1 x_0}\right)$ must be hardly dependent on pH. This may be ascribed to the fact that the value of c_1 is very small, or that Mechanism A is not correct as described in the text.

11) The values of pK and ΔH of the groups considered to be possible as the essential group are listed as follows, being taken from the table of Edsall (*16*).

	imidazolium	α-ammonium of cystine	α-ammonium of othe amino acids
pK (25°)	5.6–7.0	6.5–8.6	7.6–8.4
ΔH (kcal. M^{-1})	6.9–7.5		10–13

And here is made a brief remark concerning the results on the heat of activation and on the entropy of activation: As evident from Table I, there are considerable differences among these values of the three turnip peroxidases, indicating that these peroxidases are clearly of different kinds. Comparing with those of HRP, it may be inferred that those of TP-D are the most similar to those of HRP.

SUMMARY

1. The values of the rate constant of the reaction of formation of Complex I (k_1), and those of the rate constant of the reaction of Complex II with hydrogen donor (k_d) were obtained at various pH's and temperatures, by the method of over-all reaction kinetics using turnip peroxidases A_1, A_2 and D.

2. The value of k_d was found to be dependent on the hydrogen ion concentraion of the medium. By analyzing the results obtained, it was concluded that ionization groups of the enzymes having the pK values of 7.8, 6.9 and 7.8 at 20° are essential for the reaction between guaiacol and Complex II of turnip peroxidases A_1, A_2 and D, respectively. The group was inferred, at least in the case of turnip peroxidase D, to be an α-ammonium group of a certain amino acid in the enzyme protein, taking into account the value of the heat of ionization of the group obtained from the results of the experiment on the effect of temperature upon the ionization constant.

3. The value of k_1 of turnip peroxidase D was almost constant in the pH region 6~9, but decreased with the decrease in pH below 6.

4. The value of the heat of activation and that of the entropy of activation were obtained concerning with the reaction of formation of Complex I of turnip peroxidase D, and the reactions of Complex II of turnip peroxidases A_1, A_2 and D with guaiacol.

The author is indebted to Profs. B. Tamamushi, and Y. Ogura of the University of Tokyo, and Prof. N. Ui of the Gunma University for their advice and encouragement. This study was aided in part by a Grant-in-Aid for Scientific Research from the Ministry of Education given to the Research Group on "Mechanism of Enzyme Action."

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

INVESTIGATION ON CAERULOPLASMIN

I. PURIFICATION AND SOME PHYSICO-CHEMICAL PROPERTIES OF CAERULOPLASMIN

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(Received for publication, February 1, 1960)

Caeruloplasmin was first reported by Carl G. Holmberg and C. B. Laurell (*1*), who investigated its properties and enzyme activity, which they regarded as being identical with that of laccase (*2*). However, the preparation they handled contained some impurities, so that the physico-chemical properties as well as the physiological significance they attributed to caeruloplasmin remained a subject of reinvestigation. We purified this enzyme to a satisfactory degree by fractionating with ammonium sulfate and by zone electrophoresis. Herein are reported the experimental results dealing with the physico-chemical properties of thus purified caeruloplasmin.

PREPARATION OF CRUDE CAERULOPLASMIN

The starting material used was porcine blood, which was first treated according to Holmberg (*1*), (with some modifications) as described in the flow sheet given in Scheme I. The electrophoretic and ultracentrifugal patterns of the crude sample thus obtained (Fraction III) showed several peaks, so that further purification was attempted by the following procedure.

METHOD OF PURIFICATION

The procedure consisted of three steps: removal of impurities by heat denaturation, fractionation with ammonium sulfate and zone electrophoresis. The purification procedures in the first and second steps are given in Scheme II. The blue precipitate obtained in the second step of purification (Fraction VI) was dissolved in a minimum volume of water and then dialyzed against veronal buffer (pH 8.5, ionic strength, $\mu=0.1$).

Since the solution obtained in the second step (Fraction VI) still contained a small amount of colorless impurities, it was further purified by means of zone electrophoresis ($2V\ cm^{-1}$) using a cellulose powder column imbued with a veronal buffer of pH 8.5, $\mu=0.1$. A blue zone moved on the column, and, after 17 hours of electrophoresis, the blue substance was removed from the supporting medium. The solution of this substance was dialyzed against veronal buffer (pH 8.5, $\mu=0.1$). This sample gave a single peak in both electrophoretic and ultracentrifugal tests, as shown in Figs. 1 and 2. The physico-chemical and chemical properties of caeruloplasmin described in this report were observed with this sample. Trials of crystallization of caeruloplasmin from this sample with ammonium sulfate or alcohol at various pH were unsuccessful.

Scheme 1

Flow Sheet for Preparation of Crude Caeruloplasmin

1000 ml. of Porcine Serum

9000 ml. of distilled water added. pH adjusted to 6.2 and centrifuged at $10^4 \times g$, 5 min. at 10°.

Precipitate (Discarded)

Supernatant

pH adjusted to 5.2 and alcohol added to a concentration of 15% (v/v), at 0°. Centrifuged at $10^4 \times g$, 5 min., at 0°.

Blue-green Precipitate (Fraction I)

Supernatant (Discarded)

Dissolved in 0.9% NaCl solution, and dialyzed against water below 20°.

The Dialyzed Solution (250 ml.)

pH adjusted to 6.5, equal volume of 1 : 9 mixture of chloroform-alcohol added, and centrifuged at $3500 \times g$, 5 min., at 20°.

Precipitate

Supernatant (Discarded)

Suspended in 0.9% NaCl solution, stirred, and centrifuged at $3500 \times g$, 5 min., at 20°. (This procedure was repeated several times until the blue color in the supernatant had disappeared.)

Precipitate (Discarded)

Supernatant combined (Fraction II)

Dialyzed overnight, pH adjusted to 5.5, added equal volume of 1 : 9 mixture of chloroform-alcohol in the same way as before. Centrifuged at $3500 \times g$, 10 min., at 15°.

Precipitate

Supernatant (Discarded)

Extracted with NaCl solution, and dialyzed over-night against distilled water. Small amount of colorless precipitate discarded by centrifugation.

Crude Preparation of Caeruloplasmin (Fraction III)

Scheme 2

Flow Sheet or Purification of Caeruloplasmin (the First and Second Steps)

100 ml. of Crude Caeruloplasmin Solution (Fraction III)

1 g. NaCl added, kept in a water bath of 70°, 5 min. After cooling, centrifuged.

Precipitate (Discarded) — Supernatant

Saturated ammonium sulfate solution added up to a concentration of 35%, and centrifuged.

Precipitate (Discarded) — Supernatant

Concentration of $(NH_4)_2SO_4$ increased to 45%, and centrifuged.

Precipitate (Discarded) — Supernatant

Concentration of $(NH_4)_2SO_4$ increased to 48%, and centrifuged immediately.

Precipitate (Discarded) — Supernatant

pH adjusted to 5.1, and left standing over-night at 5°. Centrifuged at $3500 \times g$, at 0°.

Precipitate (Discarded) — Snpernatant

Left standing for several days at 0°. Centrifuged.

Deep Blue Precipitate (Fraction IV) — Slightly Blue Supernatant (Discarded)

Dissolved in a minimum volume of water and dialyzed against distilled waetr at 0°.

The Dialyzed Solution

pH adjusted to 5.1, and saturated ammonium sulfate solution added until the solution became slightly turbid. Left standing over-night at 0°. Centrifuged at $3500 \times g$, 10 min., at 0°.

Precipitate (Discarded) — Supernatant

Left standing several days at 0°, and centrifuged at $3500 \times g$, 10 min., at 0°.

Deep Blue Precipitate (Fraction V) — Supernatant (Discarded)

The procedure from Fraction IV to V was repeated 3 times in exactly the same way.

Fraction VI

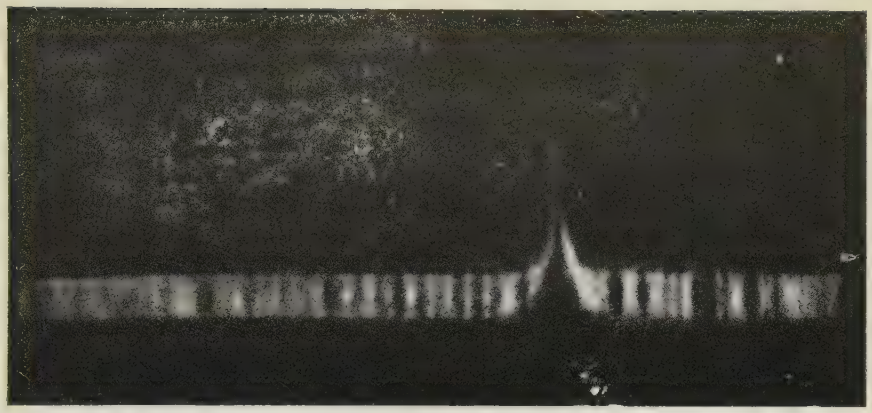

FIG. 1. Electrophoretic pattern (ascending) of caeruloplasmin; veronal buffer, pH 8.5, μ=0.1 and protein concentration=2%. The photograph was taken 70 min. after the beginning of electrophoresis.

FIG. 2. Sedimentation pattern of caeruloplasmin; 0.9% NaCl solution, pH 6.7, μ=0.15, and protein concentration=1.2%. The picture was taken 48 min. after the rotor attained full speed of 59780 r.p.m.

PHYSICO-CHEMICAL PROPERTIES OF PURIFIED CAERULOPLASMIN

1. Molecular Weight—The molecular weight of the purified caeruloplasmin was determined by measuring the sedimentation and diffusion constants. Sedimentation coefficients were measured in a Spinco Ultracentrifuge, Model E, at 59780 r.p.m. The sample solutions for observations were prepared by dissolving caeruloplasmin in 0.9 per cent sodium chloride solution, pH 6.7, μ=0.15. Corrections were made for the differences in temperature and the medium used, in order to estimate sedimentation coefficients ($S_{w,20}$), which are expressed in Svedberg units in this report. The $S_{w,20}$ value at zero protein concentration (*sedimentation constant*) was estimated by extrapolation

from the $S_{w,20}$ values at various protein concentrations. The partial specific volume assumed for the calculation was 0.75.

In FIG. 3, the $S_{w,20}$ values observed for 0.9-0.15 per cent caeruloplasmin solution are plotted against protein concentration. The sedimentation constant estimated from the data was 7.6 *S*. This value is larger than the value, 7.2 *S*, the coefficient reported by Holmberg and Laurell (*1*), which was obtained with their preparation including impurities.

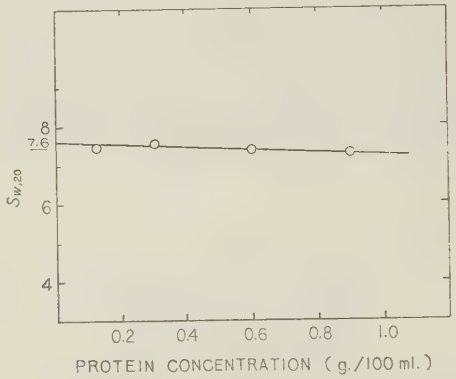

FIG. 3. Extrapolation of $S_{w,20}$ to zero protein concentration.

Diffusion coefficients ($D_{w,20}$) were measured in a Hitachi Diffusion Apparatus equipped with a Philpot-Svensson type of Schlieren optical system, using a cell of Neurath's type. From the $D_{w,20}$ values at various concentrations of caeruloplasmin, the diffusion constant was evaluated by extrapolation. The diffusion constant thus obtained was $4.4 \pm 0.01 \times 10^{-7}$ cm^2 sec^{-1}. The molecular weight and the frictional ratio of the purified caeruloplasmin, therefore, were found to be $162{,}000 \pm 1700$ and 1.31, respectively. This molecular weight is appreciably larger than the previously reported value, 151,000 (*1*). If we may assume a prolate ellipsoidal molecule with 30 per cent hydration for the molecule of caeruloplasmin, the axial ratio (a b) is estimated to be 3.6.

2. The Absorption Spectra of Caeruloplasmin—The absorption spectrum of the purified caeruloplasmin was observed with a Cary Recording Spectrophotometer, Model 14 M. In FIG. 4 are reproduced the results obtained which showed two strong bands at 279 and 610 mμ with a shoulder near 410 mμ. The two strong bands were similar in shape and position to those of laccase reported by Nakamura (*3*), but the shoulder of caeruloplasmin appeared at 410 mμ, compared with 440 mμ observed for the corresponding shoulder in the case of laccase.

The molar extinction coefficient (ε) of copper at 610 mμ, which was calculated from the difference between the absorbance at 610 mμ of blue caeruloplasmin and that of leuco form prepared by adding ascorbic acid, was $1.40 \pm 0.11 \times 10^3$ cm^{-1} *M* of copper. The copper content of caeruloplasmin

used for the calculation was measured colorimetrically at 440 mμ by the diethyl-dithiocarbamate method using *iso*amyl alcohol as the solvent. The ε value thus determined was unusually higher than that of simple Cu^{++}

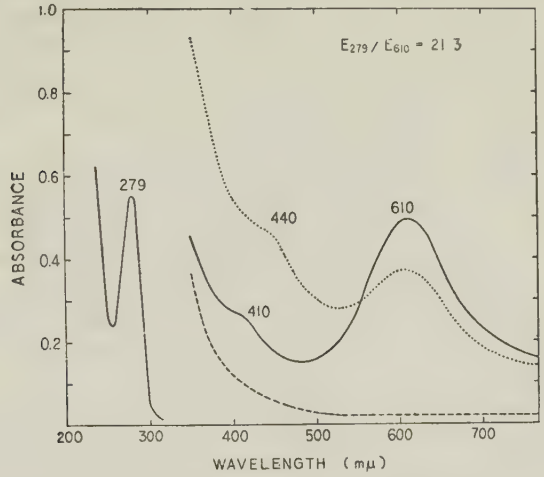

FIG. 4. Absorption spectra of caeruloplasmin in NaCl solution (pH 6.7, μ=0.15).

——; Spectrum of native caeruloplasmin.

----; Spectrum of leuco caeruloplasmin prepared by adding ascorbic acid.

····; Spectrum of caeruloplasmin with diethyl-dithiocarbamate (0.005%). The increase of absorbance at shorter wavelengths was caused by turbidity.

compounds which is rarely greater than 200. The anormalously high value of ε will be discussed later together with the data obtained with copper chelating reagents.

3. Chemical Properties of Caeruloplasmin—Pure caeruloplasmin was gradually decolorized when dialyzed against distilled water, and copper ion was detected in the dialyzing solution. The electrophoretic pattern of the partially decolorized caeruloplasmin thus obtained showed at least three peaks at pH 7.0, μ=0.05, and the peak having the slowest mobility was blue. However, no change was observed in its ultracentrifugal analysis. These findings suggest that the ionic strength is important to maintain the native structure of caeruloplasmin.

Caeruloplamin was hydrolyzed with diluted hydrochloric acid and the sugars in the hydrolysate were separated by both paper chromatographic and paper electrophoretic methods. The spots colored with aniline-phthalic acid (dissolved in butanol) proved the presence of at least two kinds of hexose and a pentose, as judged from the values of R_f and electrophoretic mobility.

Caeruloplasmin was not denatured by heating at 70° for 5 minutes (pH

6.5-8.5, μ=0.1), but was denatured at 100° with decolorization. The high stability to heat is probably due to the presence of polysaccharides as the components, considering the fact that sugar-containing proteins such as albumin, ovalbumin, laccase (*3*), and many other mucoids are known to resist to heat denaturation more strongly than do sugar-free proteins. Caeruloplasmin was not attacked by trypsin, but was digested by Pronase-P (a protease purified from *Streptomyces griseus*). The chemical constituents of caeruloplasmin and the manner in which the sugars combine with the protein moiety is now under investigation in our laboratory.

To remove copper atoms from caeruloplasmin molecule, the solution was dialyzed against 0.05 *M* cyanide. The solution which became colorless by this treatment was dialyzed against 0.9 per cent sodium chloride solution to remove cyanide ion completely. The blue color of caeruloplasmin reappeared immediately after the copper free and colorless caeruloplasmin solution thus prepared was carefully dialyzed against cuprous chloride solution. The use of excess cuprous ion in the dialysating solution was apt to denature the protein and a strong blue precipitate was formed.

The copper atoms, which were removed from the apoprotein of caeruloplasmin by the action of glacial acetic acid, reacted with both neocuproine (2,9-dimethyl-1,10-phenanthroline) and diethyl-dithiocarbamate, of which the former reacts specifically with cuprous ion and the latter with cupric one. When a neocuproine solution in glacial acetic acid was added to a caeruloplasmin solution, the reaction mixture became yellow, which indicated the presence of cuprous ion. On addition of aqueous solution of diethyl-dithiocarbamate to a caeruloplasmin solution followed by addition of glacial acetic acid, the reaction mixture became redish-yellow. These qualitative tests proved the presence of both monovalent and divalent coppers in caeruloplasmin.

In order to study the state of copper *in situ* in caeruloplasmin, 0.1 per cent aqueous solution of diethyl-dithiocarbamate was added to a caeruloplasmin solution of pH 6.0 with no addition of glacial acetic acid. The color of the mixture changed from blue to faint greenish-yellow, which contrasts with the strong redish-yellow color developed with free cupric ion. The change of the spectrum is shown in Fig. 4, which indicates the transformation of the band of the native caeruloplasmin at 610 mμ into a band at 440 mμ. When the excess of reagent was removed completely from this solution by dialysis, the blue color did not reappear immediately, and on adding acetic acid to this solution in order to cleave the copper atoms from the protein moiety, a yellow color was clearly observed which was due to the reaction between the copper ion liberated from caeruloplasmin and the reagent. These results prove that the cupric ion existing on the surface of caeruloplasmin was chelated by the reagent applied.

When a caeruloplasmin solution with diethyl-dithiocarbamate was left standing for one or two days under an aerobic condition at 15°, the solution became gradually blue in color and at the same time some yellow-colored insoluble crystals free from copper were formed. In the supernatant no

more diethyl-dithiocarbamate was detected. Under an anaerobic condition, however, the blue color did not appear and no crystalline precipitate was formed. It may be, therefore, inferred that the precipitation of the yellowish crystals may have been caused by the oxidation of diethyl-dithiocarbamate.

It was reported by Nakamura that the copper atoms of the laccase molecule (isolated from *Rhus vernicifera*) were divalent because it did not react with neocuproine. The state of the copper atoms in caeruloplasmin seems to be different from that in laccase, judging from the following results obtained in the present study: i) the copper of caeruloplasmin reacted with both neocuproine and dietyl-dithiocarbamate; ii) the addition of cuprous ion to colorless caeruloplasmin caused a reappearance of a blue color. Possibly, both cuprous and cupric forms are present in the caeruloplasmin molecule, and if we take the same point of view as proposed by Orgel (*5*), the cuprous and cupric ions may be in the state of resonance between the structures: Cu^{+}-O_2-Cu^{+}, Cu^{++}-O_2^{-}-Cu^{+}, Cu^{+}-O_2^{-}-Cu^{++}, and Cu^{++}-O_2^{--}-Cu^{++}. The resonating states of copper may also account for the high value of ε at 610 mμ of the copper atom of caeruloplasmin. The finding that dietyl-dithiocarbamate bound to the copper of caeruloplasmin was auto-oxidizable, together with the fact that the reagent is a strong inhibitor of caeruloplasmin in the oxidation of paraphenylenediamine or ascorbic acid (*2*) suggests that the enzymatic reaction may take place by the combination between the substrate and the copper atom in the caeruloplasmin molecule.

SUMMARY

Caeruloplasmin was purified by repeated fractionations with ammonium sulfate followed by zone electrophoresis. The sample thus prepared gave a single peak in both electrophoretic and ultracentrifugal tests indicating the sample was homogeneous. Sedimentation and diffusion constants observed for this sample were 7.6 *S* and 4.40×10^{-7} cm^2 sec^{-1}, respectively, from which the molecular weight of caeruloplasmin was calculated to be $162{,}000\pm1700$. Caeruloplasmin was found to be a sugar-containing protein. It contains at least three kinds of sugar: two hexoses and a pentose. Caeruloplasmin is heat stable in some conditions. The copper atom *in situ* in caeruloplasmin molecule was studied by the color reactions with neocuproine and diethyl-dithiocarbamate. The results indicated the presence of both monovalent and divalent coppers. The significance of the color reactions was discussed in relation to the activity of caeruloplasmin as an oxidase.

The author wishes to express his deep gratitude to Prof. S. Hattori, University of Tokyo and Prof. T. Sato, Prof. K. Shibata and Dr. Nagahisa, Tokyo Institute of Technology, for their kind guidance throughout this work. He is also thankful to Mr. Yoshikawa and Mr. Korida for their help in this investigation. This work was supported by a grant from the Ministry of Education.

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The Journal of Biochemistry, Vol. 48. No. 2, 1960

STUDIES ON TAKA-MALTASE

III. INHIBITION OF TAKA-MALTASE I BY CARBOHYDRATES

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(Received for publication, February 8, 1960)

As reported in the previous papers (*1*, *2*), Taka-maltase (α-glucosidase) was isolated by means of the weak anion exchange resin (Duolite A-2) column chromatography from the supernatant of rivanol fractionation in the course of purification of Taka-amylase A from taka-diastase, and optimum pH for its activity, stability and substrate specificity were studied.

For the purpose of examining the affinity of Taka-maltase I for substrates, Michaelis' constants for maltose and α-phenyl-D-glucoside, the inhibitor constants of various carbohydrates and modes of inhibition were studied by means of Lineweaver-Burk's plot.

MATERIALS AND METHODS

Enzyme—Enzyme solution of Taka-maltase I, which was purified according to the procedure described in the previous paper (*1*), was used in the present experiment.

Assay—A half ml. of enzyme solution, 1.0 ml. of substrate solution, 1.0 ml. of 0.5 *M* acetate buffer solution of pH 4.7 and 0.5 ml. of inhibitor solution or water were mixed and kept at 30°. As the substrate, 0.0004–0.002 *M* solution of maltose and 0.001–0.01 *M* solution of α-phenyl-D-glucoside were used. Concentration of the inhibitor solution was 0.05–0.2 *M*.

In case of maltose, the increase in reducing power in 10 minutes was measured by Somogyi-Nelson's colorimetry (*3*, *4*) and in case of α-phenyl-D-glucoside, amount of liberated phenol in 30 minutes of reaction was estimated by Folin's phenol reagent (*5*).

Obtained data were plotted according to Lineweaver-Burk (*6*) placing 1/v on the longitude and 1/S on the abscissa, and Michaelis' constant (Km), maximum Velocity (Vmax) and inhibitor constant (Ki) were calculated.

RESULTS AND DISCUSSION

Km and Vmax—Table I shows Michaelis' constants (Km) and maximum velocities (Vmax) of Taka-maltase I in hydrolysing maltose and α-phenyl-D-glucoside, which obtained according to the Lineweaver-Burk's plot. Taka-maltase I showed stronger affinity for α-phenyl-D-glucoside than for

maltose, although it hydrolysed maltose more rapidly than α-phenyl-D-glucoside.

TABLE I

Km and Vmax of Taka-Maltase I

Substrate	Km	Vmax
	M	μmole/hr./$E_{280\,m\mu}$
Maltose	3.5×10^{-3}	1300
α-Phenyl-D-glucoside	9.3×10^{-4}	15

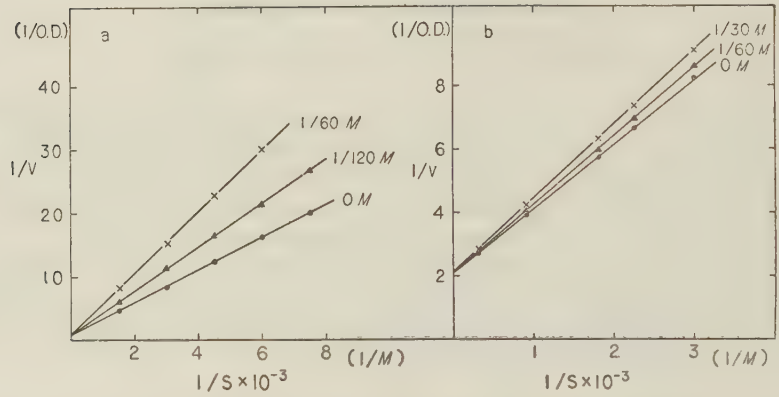


FIG. 1. Lineweaver-Burk's plots of the competitive inhibition of Taka-maltase I by carbohydrates. (a) Trehalose-inhibition of hydrolysis of maltose and (b) sucrose-inhibition of hydrolysis of α-phenyl-D-glucoside.

Ki—The following carbohydrates and glucoside, such as sucrose, raffinose, D-xylose, α-methyl-D-glucoside, trehalose and D-glucose showed competitive inhibition (see Fig. 1) and their Ki are shown on the Table II. Such compounds as lactose, cellobiose, D-galactose, D-mannose, rhamnose, mannitol, sorbitol and inositol neither inhibited the hydrolysis of α-phenyl-D-glucoside nor were hydrolysed detectably by the enzyme under the condition of the assay.

The substrates for Taka-maltase I have common configuration of α-glucosyl residue (maltose, α-methyl-D-glucoside, α-phenyl-D-glucoside, α-methyl-maltoside and α-phenyl-maltoside), and the competitive inhibitors are substances bearing α-glucosyl residue similiar to the substrate (sucrose, trehalose and D-glucose) or carbohydrates of analogous configuration with α-glucosyl residue except the structure on C_6 (raffinose and D-xylose). From these facts, hydroxyls on C_1 to C_4 of α-glucosyl residue might be considered to participate in the formation of hydrogen bonds between Taka-maltase I and the substrate. The structure of maltose with affinity for the enzyme and that of cellobiose without affinity for the enzyme display that C-O bond on C_1 must have α-configuration in order that interaction between the

enzyme and the substrate takes place. Taka-maltase I has no affinity for D-mannose and D-galactose, in which the configuration of hydroxyl group on C_2 (D-mannose) or C_4 (D-galactose) is opposite to that of D-glucose. Therefore,

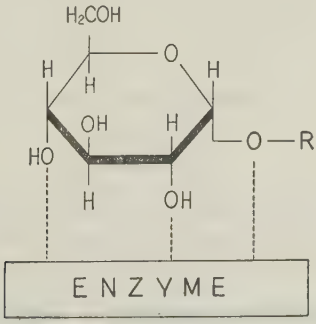
TABLE II

Mode of Inhibition and Ki of Inhibitors for Taka-Maltase I

Substrate	Inhibitor	Inhibition	Ki
Maltose	Trehalose	Competitive inhibition	2.3×10^{-2} M
	α-Methyl-D-glucoside	,,	3.9×10^{-2}
	Raffinose	,,	1.5×10^{-1}
α-Phenyl-D-glucoside	D-Glucose	Competitive inhibition	4.7×10^{-3}
	D-Xylose	,,	1.4×10^{-1}
	Sucrose	,,	$1.8\times10^{+1}$
	Cellobiose	No inhibition	
	Lactose	,,	
	D-Mannose	,,	
	D-Galactose	,,	
	Rhamnose	,,	
	Mannitol	,,	
	Sorbitol	,,	
	Inositol	,,	

hydroxyls on C_2 and C_4 of α-glucosyl residue should be considered to participate in the combination with enzyme. Weak affinity of Taka-maltase I for raffinose and D-xylose, in which hydroxyl on C_6 of glucoside is substituted or C_6-carbon is eliminated, suggests that hydroxyl on C_6 of α-glucosyl residue should participate little in the interaction between the enzyme and the substrate.

The mode of combination of Taka-maltase I with its substrate could be suggested to take place as illustrated in the following figure;



Inhibition by Sucrose—Fig. 2 shows the curves which plotted sucrose-inhibi-

tion of hydrolysis of maltose after Lineweaver-Burk's way. The mode of inhibition by sucrose was neither competitive nor non-competitive. The

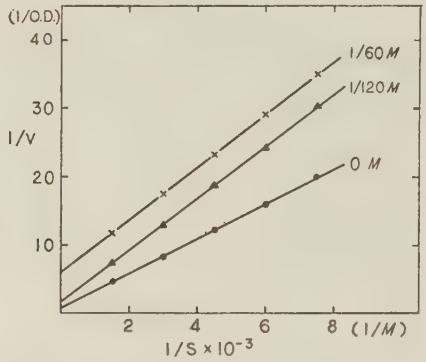


Fig. 2. Lineweaver-Burk's plot of the sucrose-inhibition of hydrolysis of maltose by Taka-maltase I.

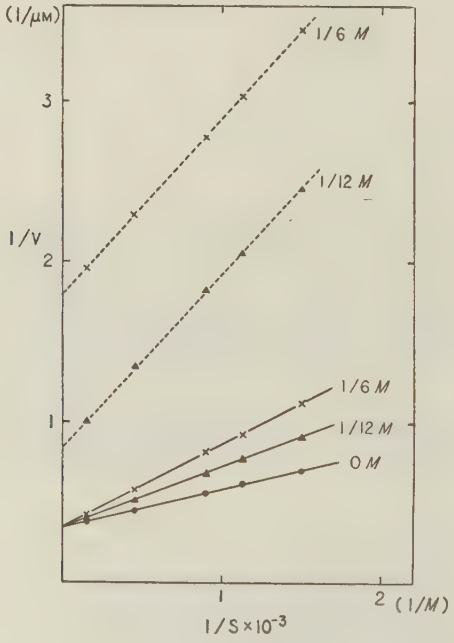


Fig. 3. Lineweaver-Burk's plot of the sucrose-inhibition of hydrolysis of α-phenyl-D-glucoside by Taka-maltase I. Solid line was the liberation of phenol and dotted line was the liberation of glucose.

curves which were obtained with addition of inhibitor paralleled with each other where the uncompetitive inhibition was suggested, but they did not

parallel with the curve which was obtained in the absence of inhibitor. This did not mean the uncompetitive inhibition.

In order to investigate this abnormality, the inhibition of Taka-maltase I by sucrose was further studied, using α-phenyl-D-glucoside as substrate (see

TABLE III

Sucrose-Inhibition of Hydrolysis of α-Phenyl-D-Glucoside

Conc. of sucrose	Conc. of α-phenyl-D-glucoside	Liberation of phenol	Liberation of glucose	Transfer
M	*M*	μmole	μmole	%
	0.0067	2.63	—	—
	0.0022	2.22	2.17	2.3
0	0.0011	1.78	1.75	1.7
	0.00089	1.62	1.60	1.2
	0.00067	1.39	1.38	0.7
	0.0067	2.43	1.00	60
	0.0022	1.94	0.74	62
1/12	0.0011	1.44	0.55	62
	0.00089	1.29	0.49	62
	0.00067	1.10	0.41	63
	0.0067	2.39	0.51	79
	0.0022	1.73	0.45	74
1/6	0.0011	1.22	0.36	70
	0.00089	1.07	0.33	69
	0.00067	0.88	0.29	67

Fig. 3). When hydrolysis was estimated through the liberation of glucose, the mode of inhibition coincided with none of any well known cases, but it was wholly a competitive inhibition when it was estimated through the liberation of phenol. When these estimations were compared with each other, the amount of liberated glucose was smaller than that of liberated phenol. The results obtained must indicated that the transfer reaction took place to considerable extents (see the Table III). The apparent abnormality can be explained by the transfer reaction which brings about the liberation of phenol but a little increase of free glucose.

SUMMARY

Inhibition of Taka-maltase I by carbohydrates was studied, and Michaelis' constants and maximum velocity of the enzymatic action on maltose and α-phenyl-D-glucoside were obtained.

Sucrose, raffinose, D-xylose, trehalose and D-glucose were competitive inhibitor, while cellobiose, lactose, D-mannose, D-galactose, rhamnose, mannitol,

sorbitol and inositol did not inhibit the enzymatic action. And the affinity of Taka-maltase I for substrates was discussed.

The authors wish to express their gratitudes to the Sankyo Co., Ltd. for their kind supply of " Takadiastase Sankyo ".

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

MECHANISM OF CYANIDE RESISTANCE IN ACHROMOBACTER

IV. CYANIDE RESISTANT RESPIRATION OF ANAEROBICALLY CULTIVATED CELLS

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(Received for publication, February 12, 1960)

In the previous paper (*1*) it was reported that *Achromobacter* strain 7 cultivated under anaerobic condition contained higher concentration of cytochrome a_2 than that cultivated under aerobic condition and that the respiration of the cells grown anaerobically was less sensitive to cyanide than that of the cells grown aerobically.

Many studies have been reported concerning the relation between the concentration of oxygen or iron in the medium and the concentration of cytochrome a_2 in the cells (*2*) (*3*) (*4*). However, these authors failed to find out the parallelism between the cytochrome a_2 content and respiratory activity of bacteria, and they threw doubt on the function of cytochrome a_2 as a cytochrome oxidase.

In this paper, studies were carried out for the purpose of finding out the relation between the cytochrome a_2 content and the respiratory activity in the presence or the absence of cyanide, of the cells grown under different oxygen concentrations. Studies on these relations are considered to be important, because it may make clear the physiological significance of cytochrome a_2 in the respiratory chain, as well as it may confirm the view on the mechanism of cyanide resistance in the bacteria (*5*).

EXPERIMENTALS

The methods of measurement of succinic oxidation activity of the intact cells, measurement of DPNH oxidase activity of cell-free preparation and measurement of absorption spectrum of cytochromes were similar to those described previously (*5*).

Cultivation of Bacteria—Bacteria were cultivated in a simple apparatus designed for this study. The apparatus was shown in Fig. 1. The cultivation vessel (capacity 6 liters) contained 4 liters of a medium with a few drops of silicon oil as an antiforming agent. Air was bubbled from the "porous air stone" which is usually used for fish-breeding. This apparatus could be easily autoclaved. In this apparatus, the medium could be kept under very aerobic condition by passing about 7 liters air per minute. When it was necessary to measure the degree of aeration, the out-letting air was led into a graduated cylinder which had stood upside-down and had been filled with water. Using this ap-

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paratus, bacteria were cultivated in a nutrient broth (pH 7.0) containing 1 per cent of meat extract, 1 per cent of polypeptone and 0.5 per cent of sodium chloride, at 32°. The rate of bacterial growth was measured nepherometrically by sampling 10 ml. of culture medium.

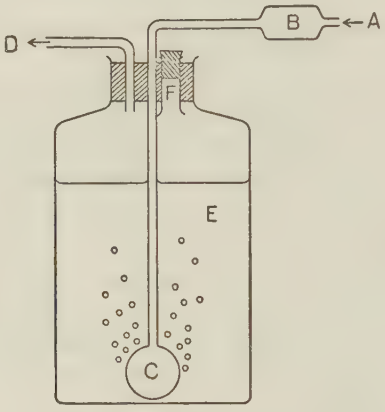

Fig. 1. Apparatus for bacterial cultivation.
A; Air compressor. B; Cotten air filter. C; Porous air stone. D; Out let. E; Medium. F; Sampling tube. For use, the apparatus was soaked in a thermostat.

Preparations from Bacterial Cell—The bacteria grown under various conditions were harvested by continuous centrifugation, washed once with 0.5 per cent saline solution. The respiratory activity of intact bacteria was measured by suspending the washed cell in $M/15$ phosphate buffer (pH 7.4). Then the washed cells were suspended in a small amount of $M/15$ phosphate buffer (pH 7.4) and this thick suspension was exposed to sonic oscillation (9–10 KC) for 15 minutes. The sonicate thus obtained was centrifuged at $5{,}000\times g$ for 20 minutes to remove the intact cells and cell debris. The turbid supernatant fluid was diluted and used for the measurement of DPNH oxidase activity. The non-diluted turbid fluid was further centrifuged at $145{,}000\times g$ for 60 minutes to obtain a clear supernatant fluid and a particulate fraction. The particulate preparation was again suspended in a small volume of $M/15$ phosphate buffer and was used for the estimation of the amount of cytochromes.

RESULTS

The bacteria were cultivated under two different air flow rates, *i.e.* about 7 liters per minute and about 200 ml. per minute. The growth curves in these cultivations were shown in Fig. 2. In both cases, cells were harvested when the number of cells reached to about 7×10^8 per ml. Hereafter these two kinds of cells were called "the aerobic cells" and "the anaerobic cells" respectively. Using these two kinds of cells, the respiratory activity, the insensitivity of respiration to cyanide and the cytochromes content were measured. The results were shown in Table I and Fig. 3. Though there was no significant difference between the respiratory activity of both kinds

of cells, the succinic oxidation activity of the anaerobic cells was much less sensitive to cyanide than that of the aerobic cells. In the case of DPNH

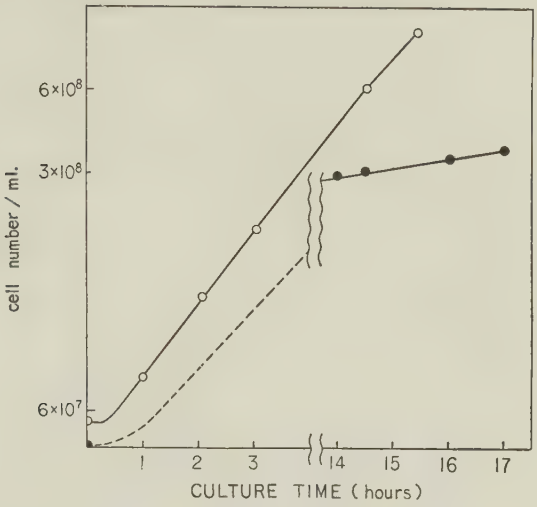

FIG. 2. Bacterial growth curve.

Bacteria were cultivated in nutrient broth at 32° using the apparatus shown in Fig. 1.

—○— aeration; 7 liters per minute.
—●— aeration; 0.2 liters per minute.

TABLE I

Effect of Cyanide on Respiratory Activities of Aerobic and Anaerobic Cells

	Succinic oxidizing activity of intact cell (Q_{O_2} μl./hr./10^8 cell)			DPNH oxidase activity ($-\Delta OD_{340m\mu}$/min./mg.N)		
	−KCN	+KCN (10^{-3} *M*)	Inhibition %	−KCN	KCN (10^{-3} *M*)	Inhibition %
aerobic cell	11.3	0.9	92	2.6	0.5	81
anaerobic cell	9.9	6.2	38	5.4	3.1	43

Succinic oxidizing activity of intact cell was measured using Warburg respirometer. Each Warburg vessel contained about 4×10^9 cells, 1.6 ml. of *M*/15 phosphate buffer (pH 7.4) and 0.2 ml. of 10^{-2} *M* KCN or the buffer in the main compartment, 0.2 ml. of 2×10^{-1}*M* succinate in the side arm and 0.2 ml. of 20% KOH in center well. Total volume 2.2 ml.

DPNH oxidase activity of sonicate was measured using Hitachi Photoelectric-spectrophotometer. Each photo-cell contained 0.2 ml. of sonicate, 2.3 ml. of *M*/15 phosphate buffer and 0.3 ml. of 10^{-2}*M* KCN or the buffer. Reaction was started by addition of 0.4 μ*M* of DPNH in 0.2 ml. of water. Total volume 3.0 ml.

xidase of the sonicate, the oxidase activity of the aerobic cells was lower than that of the anaerobic cells, and there was a remarkable difference

between the oxidative activity of aerobic cells and that of anaerobic cells in the presence of cyanide. As shown in Fig. 3, the concentration of cytoch-

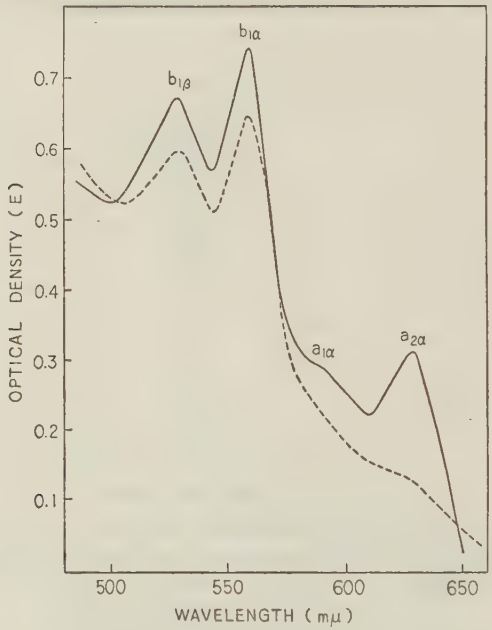

FIG. 3. Absorption spectrum of particulate preparation reduced with dithionite.

--- Preparation obtained from aerobic cells.

—— Preparation obtained from anaerobic cells.

Letters in the figure show the type of cytochrome corresponding to the peak. Nitrogen contents of crude extracts before separation of aerobic and anaerobic preparation were in the ratio 100 : 89. Spectrum was measured using Cary Model 14 Spectrophotometer, based on the opal glass method.

rome a_2 in the anaerobic cells was much higher than that in the aerobic cells, whereas, no significant difference was observed between the amount of cytochrome b_1 of these two kinds of cells. These results were quite similar to those obtained with the cells aerobically grown in presence or absence of cyanide (*5*).

When the cells which had been cultivated under anaerobic condition were further cultivated under aerobic condition, the rapid growth was observed. The changes of the properties of the respiratory activity and the amount of cytochromes were studied under these conditions. To perform this experiment in a steady state, a cultivation of bacteria was carried out as follows. As shown in Fig. 4, from the 4 liters of the anaerobic culture medium, 2 liters of the medium were replaced by the equal volume of fresh medium, and the culture condition was changed to aerobic one. When the

amount of bacteria in the medium was doubled, 2 liters of the medium was again replaced by the fresh medium and the cultivation was carried on. The same treatment was repeated twice more. From each two liters of the

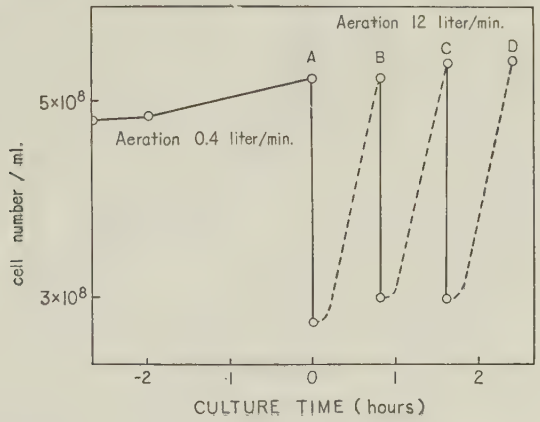

FIG. 4. Bacterial growth curve.

From the 4 liters of the anaerobic culture (aeration; 0.4 liter/min.), 2 liters was replaced by the equal volume of fresh medium at A followed by rapid aeration (12 liter/min.). When the cultiwas vation reached to B, 2 liters of the culture medium were again replaced by equal volume of fresh medium. The same treatment was repeated at C and D. The bacteria in the culture media removed at A, B, C and D were used for the experiments shown in Table II and Fig. 5.

Medium; nutrient broth. Temperature; 32°.

TABLE II

Effect of Cyanide on Succinic Oxidizing Activity of Intact Cell

	Succinic oxidizing activity (Q_{O_2} μl./hr./10^8 cells)		
	−KCN	+KCN($10^{-3}M$)	Inhibition %
A	12.6	3.1	74.6
B	12.6	1.8	85.8
C	12.4	1.3	89.5
D	12.3	0.9	92.7

A, B, C and D in Column 1 show the intact cells harvested at the point A, B, C and D in Fig. 4, respectively. The conditions of measurement of succinic oxidizing activity were the same those given in Table I.

medium removed from the vessel the bacterial cells were harvested by centrifugation and used for experiments. In Table II, the oxidase activity and their sensitivity to cyanide were shown. Though the respiratory activity

of the intact cells remained constant throughout the course of this experiment, the succinic oxidizing activity in the presence of cyanide was decreased in

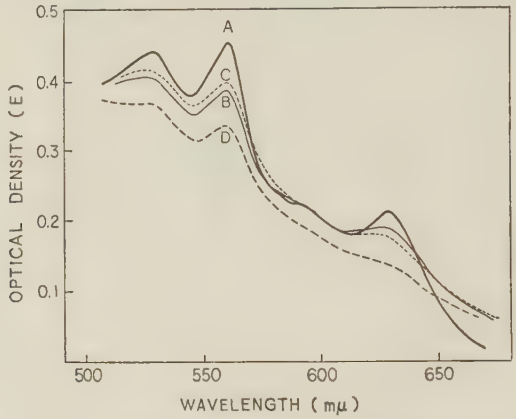

FIG. 5. Absorption spectrum of particulate preparation reduced with dithionite.

A, B, C and D show the spectrum of particulate preparation obtained from the cells harvested at the points A, B, C and D in Fig. 4, respectively. Nitrogen contents of crude extracts before separation of the particulate preparation of A, B, C and D were in the ratio 100 : 83 : 105 : 100. Spectra were measured using Cary Model 14 Spectrophotometer based on the opal glass method.

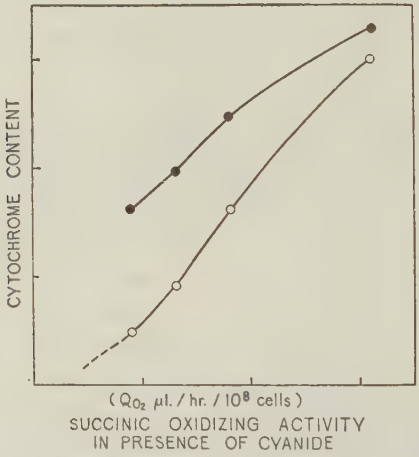

FIG. 6. Relation between cytochrome content and respiratory activity in presence of cyanide.

—●— cytochrome b_1. —○— cytochrome a_2.

Succinic oxidizing activity in presence of cyanide ($10^{-3}M$) was the value of Table II. Cytochrome content was the value of Fig. 5. Measurement of cytochrome content; see text.

reverse propotion to the bacterial growth, and at the end of this experiment the properties of the respiratory system reached to those of the aerobic cells. The absorption spectrum of the cytochromes of these cells were shown in Fig. 5. The amount of cytochrome a_2 also seemed to be decreased in reverse proportion to the bacterial growth. Though the amount of cytochrome b_1 was also changeable, it was not decreased in reverse propotion to the bacterial growth. These results strongly suggest that there is a close relation between cytochrome a_2 content and the respiratory activity in the presence of cyanide.

As it was found to be very difficult to oxidize cytochromes in the particulate preparation, the amount of cytochrome a_2 and b_1 could not be estimated from the difference spectrum (reduced minus oxidized). Therefore, the amount of cytochrome a_2 and b_1 was estimated as follows. First, straight lines were drawn between 545 mμ and 580 mμ, and between 600 mμ and 660 mμ on the spectrum. Then the amount of cytochrome a_2 and b_1 was estimated from the difference (spectrum minus straight line) in optical density at 630 mμ and 561 mμ, respectively. Fig. 6. shows the relation between the amount of cytochrome a_2 and the respiratory activity in the presence of cyanide. A parallelism was observed between them.

DISCUSSION

Many studies have been reported concerning the relation between the cytochrome a_2 content and cultivation condition. Moss (*2*) reported that *Escherichia coli* grown under anaerobic condition contained higher concentration of cytochrome a_2 than that grown under aerobic condition, whereas there was no significant difference between the respiratory activity of the two kinds of cells, and he threw doubt on the physiological importance of cytochrome a_2 as the terminal oxidase. After that Moss (*3*) also reported a marked dependence of cytochrome a_2 content of *Aerobacter aerogenes* on oxygen concentration in the medium, and supported the view that the cytochrome a_2 was the oxidase. On the other hand, Tissieres (*4*) found out that though the cytochrome a_2 content of *Aerobacter aerogenes* was decreased by iron deficiency, the respiratory activity of the cells was almost equal to that of iron rich cells. From this result, he also threw doubt on the role of cytochrome a_2. Keillin (*6*) and Smith (*7*) also pointed out that there was a lack of parallelism between the cytochrome a_2 content and the respiratory activity of bacteria. So then, these authors failed to find out the relation between the cytochrome a_2 content and the respiratory activity.

In *Achromobacter* strain 7, there was no relation between the cytochrome a_2 content and the respiratory activity. However, a parallelism was observed between the cytochrome a_2 content and the respiratory activity in the presence of cyanide (10^{-3} *M*). In the previous paper (*1*), it was reported that the oxido-reduction of cytochrome a_2 was strongly but not completely inhibited by 10^{-3} *M* concentration of cyanide and increase in the amount of cytochrome a_2 caused the increase of the electron transfer capacity in the presence of cyanide at the step of cytochrome a_2. Therefore, the results presented in

this paper could be explained as follows. As the respiration of these bacteria may not be limitted by the reaction catalized by cytochrome a_2, the change in the amount of cytochrome a_2 seemed to have no influence on the respiratory activity. However, the fact that the respiratory activity was strongly inhibited by 10^{-3} *M* concentration of cyanide shows that the reaction catalized by cytochrome a_2 limits the over-all reaction in the presence of cyanide. Therefore, if cytochrome a_2 have an important role in the respiratory chain, there must be a parallelism between the cytochrome a_2 content and the respiratory activity in the presence of cyanide. The results given in Fig. 6 show the parallelism between these two.

In addition, the fact that there was a parallelism between the cytochrome a_2 content and the respiratory activity in the presence of cyanide confirmed the view on the mechanism of cyanide resistance in the bacteria (*5*).

As shown in Table II, when the anaerobic cells were cultivated under aerobic condition, the cyanide resistance in the respiration decreased in reverse proportion to the bacterial growth. This result was quite different from the case that the cells which had been made ressistant to cyanide in the presence of cyanide, was aerobically cultivated in cyanide-free medium (*5*). In the later case, the insensitivity to cyanide of the respiratory activity could be observed even after 3 or 4 times cell-divisions in the cyanide-free medium. Possible explanation is that in the former case, though the cytochrome a_2 content was indeed high in anaerobic cells, this does not mean the increase of the cytochrome a_2 forming activity, namely cytochrome a_2 seemed to be merely accumulated in the cells owing to the slow growth; whereas in the later case, the cytochrome a_2 forming activity was really increased, probably owing to the some fundamental changes in the cell constituent such as in the level of nucleic acid. This may be the reason why the cyanide insensitivity obtained in the presence of cyanide was maintained during 3 or 4 times cell-divisions in the cyanide-free medium.

SUMMARY

1. *Achromobacter* strain 7, grown under anaerobic condition was found to possess greater amount of cytochrome a_2 than that grown under aerobic condition.

2. Though the respiratory activities of both kinds of cells were quite equal, the anaerobically cultivated cells showed a higher respiratory activity than the aerobically cultivated cells, in the presence of cyanide (10^{-3} *M*); namely the respiration became cyanide resistant during an anaerobic cultivation.

3. A parallelism was observed between the cytochrome a_2 content and the respiratory activity in the presence of cyanide.

4. A physiological significance of cytochrome a_2 as respiratory carrier and the mechanism of cyanide resistance in anaerobically cultivated cells were discussed.

The authors wish to express their sincere appreciation to Dr. K. Sakaguchi for his encouragement and interest.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

STUDIES ON CYTOCHROME C_1*

I. ISOLATION, PURIFICATION AND PROPERTIES OF CYTOCHROME C_1 FROM HEART MUSCLE

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(Received for publication, February 19, 1960)

In 1940 Yakushiji and Okunuki (*1*) found in heart muscle a new cytochrome component which they called cytochrome c_1. They isolated it in a partially purified state from heart muscle, in a way similar to that of purification of cytochrome a (*1*). On the other hand, Slater (*2*) considered that cytochrome c_1 might be a denatured protein hemochromogen from the affinity of his preparation towards carbon monoxide. Recently, many observations on this component (*3–6*) have been reported and its presence and important role in the typical cytochrome system were re-established. The name cytochrome c_1, instead of cytochrome e as advocated by Keilin and Hartree (*4*), is now generally used.

To investigate the reaction mechanism of the cytochrome system of heart muscle, the purification and properties of this component have been studied by the present authors, and a part of these investigations has already been reported (*7*). It was reported that our preparation contained some flavoprotein (FAD-enzyme) and had a weak diaphorase activity. In the present paper an improved purification procedure, which gave a flavin-free preparation, and the properties of thus purified cytochrome c_1 will be reported. Purified cytochrome c_1 can be reduced with a succinic oxidase system and oxidized by a purified cytochrome system. The heme separated from the protein moiety of this component closely resembles to heme c in its spectral properties. Recently Green *et al.* (*8*) also obtained a highly purified preparation of this component by treatment with organic solvent of mitochondrial fragments from heart muscle. However, there are some differences between the properties of our purified preparation and those of Green *et al.* These differences and the activity of cytochrome c_1 as an electron carrier will be reported in detail elsewhere.

EXPERIMENTAL

MATERIALS

Cryststalline cytochrome c was prepared in its oxidized form from heart muscle accord-

* This investigation was supported in part by Research Grant RG-5871 from the Public Health Service of the National Institutes of Health, U. S. A.

ing to the method of Hagihara *et al.* (*9*) and recrystallized. The crystals were dissolved in a small amount of 0.1 *M* phosphate buffer, pH 7.4, and dialyzed overnight against the same buffer at 5°. Reduced cytochrome c was obtained by Pd-H_2 reduction.

Cytochrome a was prepared according to the method of Okunuki *et al.* (*10*). Fraction S_4 was used as a highly purified preparation of cytochrome oxidase. Fraction S_2, after being washed thoroughly with 50 per cent saturated ammonium sulfate at pH 7.4, was used as the cytochrome c-free succinic oxidase and DPNH-oxidase preparations (*11*).

Soluble succinic dehydrogenase was prepared from pig heart by the method of Wang *et al.* (*12*) with a slight modification at the extraction step as described by Keilin and King (*13*).

Yeast lactic dehydrogenase was prepared according to the method of Yamanaka *et al.* (*14*).

About 90 per cent pure DPN was prepared from baker's yeast according to the modification of the method of Okunuki *et al.* (*15*, *16*). DPNH was obtained by enzymatic reduction of DPN with crystalline alcohol dehydrogenase and ethanol according to the method of Rafter and Colowick (*17*).

Alumina C_γ gel was prepared by the method of Willstätter and Kraut (*18*). The gel was stored for 3 months before use.

Cholic acid was purified from a commercial preparation (Nissan Kenkyusho, Tokyo) and recrystallized twice from ethanol according to the method of Gattermann (*19*).

Carbon monoxide was generated by dropping formic acid into concentrated sulfuric acid at 140–160°. It was washed with saturated potassium permanganate and then with 50 per cent potassium hydroxide solution.

Other materials were obtained commercially.

METHODS

A Zeiss microspectroscope served for the estimation of the approximate location of the absorption bands. Spectra were measured by a Hitachi recording spectrophotometer, model EPS-2, and by a Cary spectrophotometer, model 14. The optical density was also measured with a Shimadzu spectrophotometer, QB-50. The reaction with carbon monoxide was measured in a Thunberg tube type cell. The concentration of cytochrome c_1 was determined spectrophotometrically from the extinction coefficients, as described later under the relevant experiments.

Protein concentration was determined either by the Biuret reaction (*20*) with blood albumin as a standard, or by the micro-Kjeldahl method in terms of nitrogen content.

Iron was determined by the method of Sandel (*21*) with a slight modification.

RESULTS

I. Purification of Cytochrome c_1 from Heart Muscle

1. Preparation of the Green Brei—This was prepared from the muscle of two beef hearts by the same method as used for the preparation of cytochrome a (*10*).

2. Extraction of Cytochromes—To 700 ml. of the Green brei was added 175 ml. of 10 per cent cholic acid in 0.1 *M* phosphate buffer, at pH 7.4. After mixing well, 190 ml. of saturated ammonium sulfate, adjusted to pH 7.4 with 4 *N* NH_4OH, was added with stirring. The mixture was left at 4° for 10

hours and then centrifuged at 30,000×*g* for 30 minutes in a Hitachi preparative ultracentrifuge, model 40P, using rotor R21. The supernatant fluid, which contained cytochromes b, c, c_1 and a little cytochrome a was carefully strained through two or three layers of cheese cloth to remove floating fat.

3. First Ammonium Sulfate Fractionation—To the extract obtained as above finely powdered ammonium sulfate was added to 55 per cent saturation and the mixture was centrifuged at 13,000×*g* for 20 minutes in the same centrifuge as used previously. Care was taken not to allow the pH to fall below 7.2. The supernatant fluid, which contained only the c-component, was discarded. The precipitate was dissolved in 2.0 per cent cholic acid in 0.1 *M* phosphate buffer, pH 7.4, and the mixture was left at 5° for about 100 hours. During this period, cytochrome b was denatured and the solution changed its color from amber to a rather milky brown and became turbid.

4. Heat Treatment and Dialysis—The mixture was centrifuged at 13,000×*g* for 25 minutes and the supernatant fluid was decanted. It was heated at 40° for 15 minutes in a water bath, and then cooled in an ice bath. The mixture was centrifuged to remove coagulated cytochrome b. The supernatant fluid contained cytochromes c_1 and a, and flavoprotein was liberated from b, c_1- complexes by this treatment. This fluid was dialyzed for about 16 hours against about 20 volumes of 0.01 *M* phosphate buffer, pH 7.4, at 5° to reduce its cholic acid concentration. By this treatment cytochrome a was precipitated and was subsequently removed by centrifugation at 13,000×*g* for 25 minutes. The supernatant was clear and orange-red in color.

5. Alumina C_γ Gel Treatment—To the above solution one tenth volume of alumina C_γ gel (23.5 mg. dry weight per ml.) was added. The mixture was left for about 30 minutes at 5°. It was then centrifuged at 3,000×g for 10 minutes and the supernatant was further treated with a half volume of the same alumina C_γ gel suspension to adsorb the component completely. The gels were combined and repeatedly washed with 0.2 *M* Na_2HPO_4 until the washings became colorlesss. By this treatment most of the flavin was removed from the preparation. The small amount of cytochrome c_1 in the washings was reserved for later purification. The washed gel was suspended in 0.1 *M* phosphate buffer, pH 7.4, and then an appropriate amount of dry starch was added to the suspension and mixed well. In a typical preparation, 30 g. of starch were added to 2 g., dry weight, of the gel. The mixture was poured into a glass column (5 cm. in diameter, 5 cm. in height) placed on a suction bottle and stirred occasionally so that it was homogeneously packed. Cytochrome c_1 was eluted from the column with a mixture of 0.1 *M* phosphate buffer, pH 7.4, saturated ammonium sulfate solution and 10 per cent sodium cholate (15 : 3 : 2 by volume). The flow rate was about 0.3 ml. per minute. The first part of eluate was colorless and then followed a reddish orange fraction with a slight opalescence which may be due to a released lipid-like substance. Then followed the main fraction which was clear red in color. The fourth fraction of the eluate, containing less of the component, was combined with the main fraction and purified further.

6. Second Ammonium Sulfate Fractionation—The combined main fraction was treated with ammonium sulfate to obtain a fraction precipitating between 35 and 55 per cent saturation. This was dissolved in 0.5 per cent cholic acid in 0.1 *M* phosphate buffer, pH 7.4. By repeating the above fractionation two or three times, a highly purified preparation of cytochrome c_1 was obtained. The fraction described in the foregoing section containing a fair amount of lipid-like substance was combined with washings reserved and also purified by ammonium sulfate fractionation, after being left for several days at 5°. On standing, a considerable amount of the substance separated out.

The highly purified cytochrome c_1 thus obtained was dissolved in 0.1 *M* phosphate buffer, pH 7.4, and used for the following experiments. To store the purified preparation, the precipitate was dissolved in 0.5 per cent cholic acid or 0.5 per cent Emasol 1130 (a non-ionic detergent) in 0.1 *M* phosphate buffer, pH 7.4, to prevent development of turbidity. A summary of the purification procedure is presented in Table I.

TABLE I

Summary of Purification Procedure

Fraction	Total volume (ml.)	Total protein[1] (mg.)	Total Cyt. c_1[2] (μmoles)	μmoles Cyt. c_1/g. protein
Cholate extract from Green brei	834	10,930	10.6	0.97
Supern. from 15% sat. $(NH_4)_2SO_4$	392	7,330	9.2	1.20
Dialysate after heat treatment	421	2,900	2.84	0.98
Alumina C_γ gel eluate	76	386	1.34	3.47
$(NH_4)_2SO_4$ fraction 30–55% sat.[3]	5.6	150	1.16	7.70

1) The values were obtained by the Biuret reaction.

2) Cytochrome c_1 was determined from the difference in absorption of the oxidized and reduced forms at 553 mμ by using the value of $E_{553m\mu}$ (Reduced—Oxidized)$=9.4\times10^3$ $M^{-1}\times$cm.$^{-1}$ (Table II).

3) The eluate was fractionated only once. Further fractionation was required to prepare the highly purified cytochrome preparation; the resulting dearease in yield was negligible.

II. Properties of Purified Cytochrome c_1

1. Absorption Spectra—The absorption spectra of purified cytochrome c_1 are shown in Fig. 1. The preparation has its absorption maxima at 553 mμ, 523 mμ and 418 mμ in the reduced form and at 523 m , 411 mμ and 278 mμ, in the oxidized form. The extinction ratios of these maxima are given in Table II.

2. Iron Content—The iron content of the highly purified preparation was estimated after repeated dialyses against 0.005 *M*. Tris-HCl brffer, pH 7.4, containing 0.05 *M* EDTA, to remove non-heme iron. From this and spectral analysis, the molar extinction coefficient was calculated, assuming that one atom of iron is contained per mole cytochrome c_1. The protein content of

the same preparation was determined by the micro-Kjeldahl method. From these results, the minimum molecular weight of the preparation was calculated to be 99,000. The results are given in Table II.

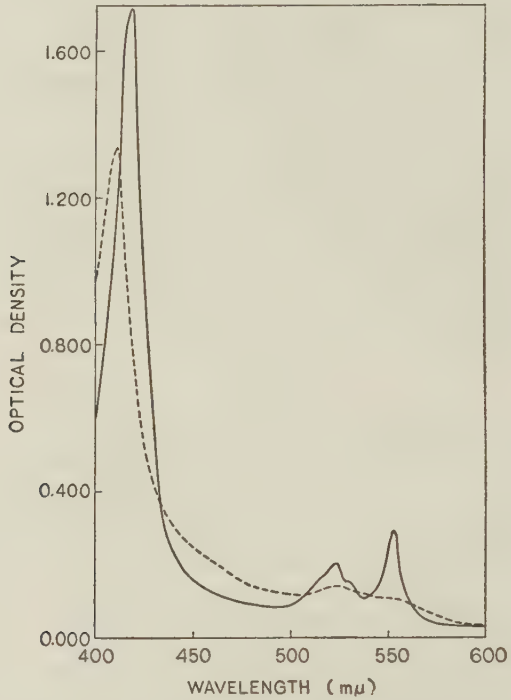

FIG. 1. Absorption spectra of cytochrome c_1.
The spectra were read in 0.1 *M* phosphate buffer, pH 7.4, by using a Cary spectrophotomer. For reduction, a trace of dithionite and for oxidition a trace of potassium ferricyanide was added. Solid line, reduced form; dotted line, oxidized form.

3. Reactivity with Carbon Monoxide, Cyanide and Azide—Slater (*2*) claimed that his preparation of c_1-component was a denatured protein hemochromogen because the absorption spectrum of the preparation changed after treatment with carbon monoxide. Therefore the effect of carbon monoxide on our purified cytochrome c_1 preparation was examined. The effect of respiratory inhibitors on cytochrome c in the physiological pH range has been studied by several investigators. Altschul and Hogness (*22*) observed that the γ-peak of reduced cytochrome c shifted from 415 mμ to 414 mμ as a result of the formation of carboxy-cytochrome c at neutral pH. Potter (*23*), Horecker and Kornberg (*24*) and Horecker and Stannard (*25*) reported that both cyanide and azide combined with cytochrome c. However, the combination of these two inhibitors with cytochrome c was considered to

be of no biological significance because of the slow formation of the cyanide compound and the large dissociation constant of the azide complex. In the present experiments, these reagents were tested with both cytochromes c and

Table II

Spectral and Analytical Properties of Purified Cytochrome c_1

Absorption maxima (mμ)	
Reduced form	553, 523, 418
Oxidized form	523, 411, 278
Extinction ratio	
	$E_{418m\mu}$ (Red.)/$E_{553m\mu}$ (Red.)=5.97
	$E_{523m\mu}$ (Red.)/$E_{553m\mu}$ (Red.)=0.67
	$E_{418m\mu}$ (Red.)/$E_{411m\mu}$ (Oxd.)=1.27
	$E_{278m\mu}$ (Oxd.)/$E_{418m\mu}$ (Red.)=1.76−1.86[1]
Extinction coefficient	$E_{553m\mu}$ (Red.)=$15.3\times10^3\,M^{-1}\times$cm.$^{-1}$
	$E_{553m\mu}$ (Red−Oxd)=$9.4\times10^3\,M^{-1}\times$cm.$^{-1}$
Iron content	10.1 μatoms per g. protein[2]

1) Values for this ratio measured with several preparations.

2) Analyses were made on duplicated samples of the most highly purified preparation. Protein contents were estimated as nitrogen content by the micro-Kjeldahl method.

c_1 under the conditions used by the above investigators. The results illustrated in Table III show that the spectrum of cytochrome c_1 is not altered by these reagents, while that of cytochrome c changes as reported by the above investigators. These results will be discussed later.

Table III

Effect of Some Chemical Reagents on Cytochromes c_1 and c

Reagent	Shift of absorption spectrum (mμ)	
	Cytochrome c[1]	Cytochrome c_1[1]
CO	415⟶414	418
KCN (0.0063 *M*)	530⟶535	523
	408⟶413	411
NaN_3 (0.1 *M*)	530⟶533	523
	408⟶410	411

1) Measured in phosphate buffer, pH 7.4.

4. Effect of Oxidizing and Reducing Agents—Reduced cytochrome c_1 could be rapidly oxidized with a trace of potassium ferricyanide. It was also oxidized at pH 3.0 in 0.5 per cent Emasol 1130. This oxidized cytochrome c_1 could be reduced enzymatically after adjusting the pH of the solution to

7.4. Cytochrome c_1 was rapidly reduced by reducing reagents such as sodium dithionite, potassium borohydride, ascorbic acid, sodium thioglycolate and cysteine. Reduced cytochrome c_1 showed scarcely any autoxidizability under normal physiological conditions. In fact, the purified cytochrome c_1 was a mixture of the reduced and oxidized forms and the reduced form could not even be oxidized by aeration. However, when air was bubbled through a dithionite-reduced preparation to remove the excess reagent, a slow oxidation was often observed in the absence of ammonium sulfate. This oxidation could be completely prevented by the presence of 15 per cent saturated ammonium sulfate. The gradual autoxidation of cytochrome c_1 seems to be due to the occurrence of some structural modification, which may be, in some way, prevented by the effect of ammonium sulfate.

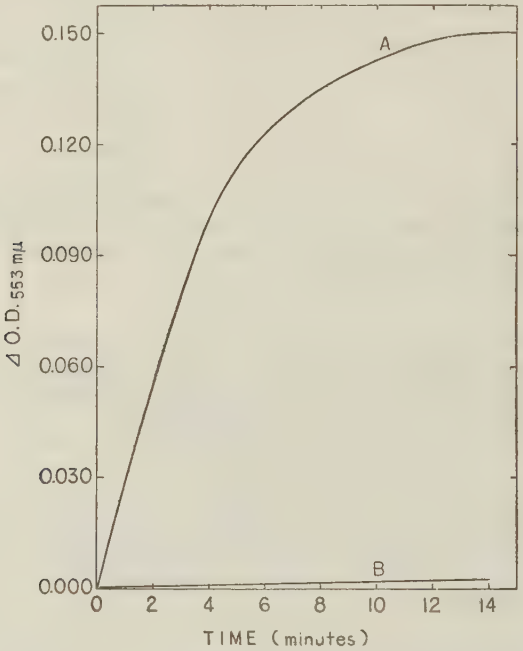

FIG. 2. Enzymatic reduction of cytochrome c_1 with succinate and fraction S_2.

The reduction was measured in a Hitachi spectrophotometer EPS-2 as increment in optical density at 553 mμ at 27°. The reaction mixture contained 0.02 *M* phosphate buffer, pH 7.4, 50 μmoles sodium succinate, 50 mμmoles cytochrome c_1, 3 μmoles potassium cyanide, and or without 50 μg. antimycin A. Curve A and B show the results obtained in the absence and presence, respectively, of antimycin A.

5. Properties as an Electron Carrier—The mode of reaction of cytochrome c_1 with cytochromes a and c, which has been dealt with in detail in our previous report (*11*), was confirmed with the present preparation. Reduced

cytochrome c_1 was slowly oxidized by cytochrome a and rapidly on addition of a small amount of cytochrome c. These results indicate that electron transfer occurs rapidly between cytochromes c_1 and c, but slowly between cytochromes c_1 and a.

Purified cytochrome c_1 was enzymatically reduced by the action of succinic oxidase system of fraction S_2. As shown in Fig. 2, this reduction was antimycin A-sensitive. A similar result was observed using DPNH as substrate. The cytochrome c_1 preparation was not reduced by the addition of succinate or DPNH alone. The preparation also showed neither diaphorase nor DPNH-cytochrome c reductase activity. Soluble succinic dehydrogenase could not link directly with purified cytochrome c_1, but was slowly reduced by the presence of a small amount of ferricyanide. Yeast lactic dehydrogenase containing the cytochrome b_2 moiety could reduce both cytochrome c and cytochrome c_1 Detailed results of these experiments on the enzymatic reduction and oxidation of purified cytochrome c_1 will be presented in the following paper of this series.

6. Stability—A highly purified cytochrome c_1 preparation was stable at 0° for a month. But it was easily denatured and coagulated by heating at 50° for 5 minutes, indicating the heat lability of the cytochrome.

III. Properties of the Heme of Cytochrome c_1

From the spectroscopic study Yakushiji and Okunuki (*1*) reported that the heme of cytochrome c_1 was heme c. However the pyridine hemochromogen, obtained from cytochrome c_1 by the addition of pyridine to an alkaline preparation and reduced with sodium dithionite, showed absorption maxima at 551 mμ, 521 mμ and 416 mμ. These are 1 mμ further to the red compared with those of the pyridine hemochromogen of cytochrome c, indicating the possibility that the heme of cytochrome c_1 may not be identical with heme c. The heme in question could not be split from the protein moiety by the acid acetone method which is often used to split off heme a or protoheme from protein. Paul (*26*) used a silver sulfate method to separate the heme of cytochrome c, which is tightly bound to the protein moiety through a thio-ether linkage. From the absorption maxima of the pyridine hemochromogens and from the resistance to acid acetone of cytochromes c and c_1, it may be assumed that the heme of cytochrome c_1 is also bound with its protein moiety through a thio-ether linkage. In fact, the separation of heme from cytochrome c_1 could be achieved by Paul's method with a slight modification as described by Morrison *et al.* (*27*) and by Postgate (*28*).

1. Separation of Heme—To 3 ml. of approximately 10^{-4} *M* cytochrome c_1 was added an excess cold acetone. The mixture was well stirred and left for 15 minutes at room temperature. It was centrifuged and the precipitate was washed three times with a chloroform-methanol mixture (2:1 v/v) at 15 minutes intervals and then with cold acetone. The acetone dried cytochrome c_1 was suspended in distilled water to a final volume of 4.0 ml. and then 0.8 ml. of glacial acetic acid and 4.0 ml. of saturated silver sulfate solution

were added. The mixture was heated to 75–80° for 45 minutes. The heme thus liberated from protein was extracted with a glacial acetic acid-ether mixtures (1 : 3 v/v) until the solvent became colorless. The extract was washed with five volumes of 5 per cent NaCl. The solvent was then evaporated *in vacuo* and the residue was neutralized with 6 *N* NaOH and diluted with 0.05 *N* NaOH.

2. *Spectral Property of the Heme*—After adjusting the pH of the above heme solution to 12.0 with 6 *N* NaOH, pyridine was added to a final concentration of 33 per cent. On reducing the mixture with sodium dithionite, dipyridine ferroporphyrin was obtained. Dicyanide ferroporphyrin was prepared by the addition of an excess potassium cyanide instead of pyridine to the alkaline heme solution. The absorption spectrum of these compounds are shown in Fig. 3, from which it appears that the heme of cytochrome c_1 is identical with that of cytochrome c.

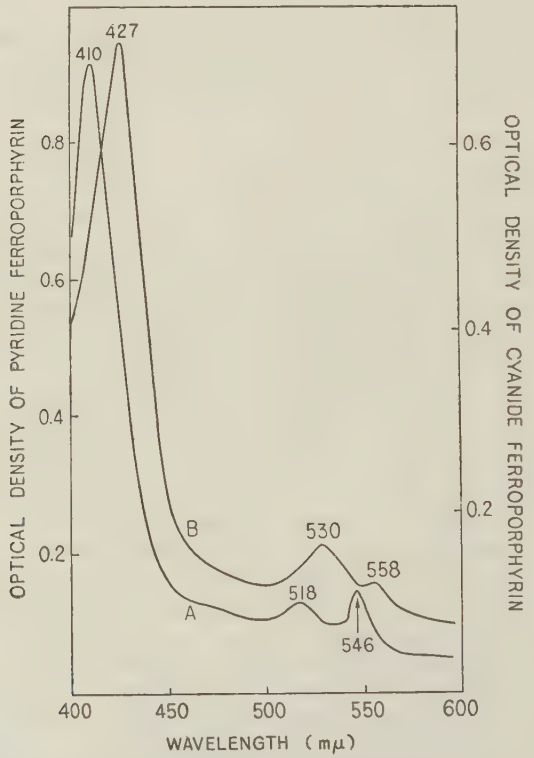

FIG. 3. Absorption spectra of dipyridine and dicyanide ferroporphyrins prepared from cytochrome c_1.

Curve A, dipyridine ferroporphyrin, Curve B, dicyanide ferroporphyrin.

DISCUSSION

Cytochrome c_1 was extracted from a particulate heart muscle preparation (the Green brei) with cholic acid and ammonium sulfate at pH 7.4. At this pH, 0.44 mg. cholic acid per mg. protein was added, while in the extraction of cytochrome a (*10*) 0.75 mg. of cholic acid has been used. The latter concentration of cholic acid was found to be equally effective for the extraction of cytochrome c_1, but 0.44 mg. per mg. protein was used here to reduce the contamination of cytochrome a in the extract. Much cytochrome b was also present in the extract, but was removed from the preparation by prolonged incubation (100 hours) and heat treatment. When incubation was omitted, or the concentration of cholic acid was 0.5 per cent or less during incubation, heat treatment was of little benefit. Addition of 0.25 *M* sucrose also stabilized the b-component. Treatment with proteinase-free lipase (*e.g.*, *Sclerotinia*-lipase) was also effective in removing the component. To remove cytochrome a, the cytochrome b-free preparation was dialyzed against phosphate buffer to reduce the concentration of cholic acid. Cytochrome c_1 is soluble even in a dilute cholate solution. It was observed that cytochrome a, which had been precipitated during this dialysis, could be recovered without loss of activity by dissolving it in the cholate solution. The gel treatment was effective in removing impurities such as flavin or lipid-like substances. Repeated fractionation with ammonium sulfate also partially removed these impurities. The gel treatment in the present method simplified the procedure in removing these impurities and increasing the yield of the purified pigment as compared with the case in our previous method.

It has been clearly established by many other investigators (*3–6*) that cytochrome c_1 occupies an important role in the typical cytochrome system. Recently, Green *et al.* (*8*) obtained a highly purified preparation of cytochrome c_1 from heart muscle and reported that their preparation was not directly linked with oxidizing and reducing systems. However, the present preparation has been shown to be coupled with a purified cytochrome oxidase system and fraction S_2 which has succinic- and DPNH-cytochrome c reductase activities. The reaction velocities of the above reactions may be expected to increase if the inhibitory effects of cholate and other impurities are eliminated from the reaction system. Such investigations are now in progress in our laboratory and will be reported in subsequent papers of this series. Except in its ability of coupling with oxidizing and reducing systems and in the calculated value of molar extinction coefficient, our preparation appears to be very similar to that obtained by Green *et al.*

Our preparation does not form a hemochromogen with carbon monoxide, indicating that the hemoprotein of the preparation is almost certainly in a native form. That the purified preparations of cytochromes c and c_1 show different reactivities towards carbon monoxide, cyanide and azide suggests that there exist some structural and functional differences between them. Since the hemes of both cytochromes have been revealed to be identical, the structural and functional differences of these cytochromes must lie in

the nature of their protein moieties. As has been shown by our experimental results, cytochrome c_1 does not react effectively with cytochrome a unless cytochrome c is provided as an intermediate carrier. On the other hand, lactic dehydrogenase from baker's yeast can link directly with both cytochrome c_1 and cytochrome c without the necessity of an intermediate. The problem as to why both cytochromes c and c_1, which are so similar in structure but not in function, are required for the terminal oxidase system, is still a matter of further investigation.

SUMMARY

1. A procedure of isolating cytochrome c_1 in a highly purified state from heart muscle is described. The procedure involves extraction with cholic acid and ammonium sulfate, fractionation with ammonium sulfate, heat treatment, adsorption with alumina C_γ gel and fractionation with ammonium sulfate.

2. The spectral properties of cytochrome c_1 and the molar extinction coefficient were measured. The preparation did not react with carbon monoxide, cyanide or azide.

3. Cytochrome c_1 can be oxidized with cytochromes a and c and reduced with succinate and DPNH in the presence of fraction S_2. The reduction is antimycin A-sensitive.

4. The heme is bound to the protein moiety by a thio-ether linkage and this linkage is split by silver sulfate treatment. The liberated heme showed the same spectral behavior as that of cytochrome c.

The authors thank Messrs. S. Takemori, J. Yamashita and K. Ohnishi for their help and discussion during this investigation.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

METABOLIC STUDIES OF BILE ACIDS

XLIII. ENZYMATIC 7-HYDROXYLATION OF 3β-HYDROXY-Δ⁵-CHOLENIC ACID

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(Received for publication, February 22, 1960)

Experiments with isotopically labeled cholesterol have shown that the bile acid is formed from cholesterol in vertebrates, such as dog (*1*), rat (*2*), and rabbit (*3*). Bergström and Lindstedt (*4*, *5*) have recently demonstrated that the nuclear transformation precedes the side-chain degradation of cholesterol in rat and that 7α-hydroxycholesterol is the immediate metabolite of this sterol.

In the present experiment, an analogue of cholesterol, 3β-hydroxy-Δ⁵-cholenic acid, which, though not shown as an intrinsic metabolite of cholesterol, was proved to be a valuable model compound for the study of cholesterol metabolism (*6*), was subjected to enzymatic hydroxylation and the formation of the supposed metabolite, 3,7-dihydroxy-Δ⁵-cholenic acid, was studied.

METHODS AND RESULTS

1. Colorimetric Determination of 3,7-Dihydroxy-Δ⁵-cholenic Acid

Bergström and Wintersteiner (*7*) reported a colorimetric determination of 7-hydroxycholesterol and related compounds, the method being based on the modified Lifschütz reaction of 'oxycholesterol.' This method has lately been used successfully, indeed, for the metabolic studies of 7-hydroxycholesterol by Yamasaki *et al.* (*8*). But it has been found that 3,7-dihydroxy-Δ⁵-steroids give a violet, but not blue color in a mixture of glacial acetic acid and concentrated sulfuric acid, when the reaction is conducted without ferric chloride, exactly like the original method and that this reaction is fairly specific and sensitive as will be indicated later. So the original Lifschütz reaction, in principle, was here used.

3,7-Dihydroxy-Δ⁵-cholenic Acid (m. p. 216~217°)—This diol was prepared from methyl 3β-acetoxy-7-oxo-Δ⁵-cholenate (m. p. 177–178°) (*9*) by potassium borohydride reduction. The details of this preparation will be reported elsewhere.

H_2SO_4-Acetic Acid Reagent—Ten ml. of conc. H_2SO_4 was mixed with glacial acetic acid to make a total volume of 100 ml.

Method—A small amount (10–50 μg. or 0.03–0.15 μmole) of 3,7-dihydroxy-Δ⁵-cholenic acid was dissolved in 0.5 ml. of glacial acetic acid, and mixed with 2.0 ml. of the H_2SO_4-acetic acid reagent. A violet color gradually developed, and after about 10 minutes, optical density of the colored solution was found to be completely stable at least for 30

minutes (Fig. 1). Fifteen minutes after the reagent was added, the extinction of the solution was read photometrically against blank, a 570 mμ filter being used. The absorption spectrum of the chromophore obtained from 3,7-dihydroxy-Δ⁵-cholenic acid shows a strong maximum at 579 mμ (Fig. 2).

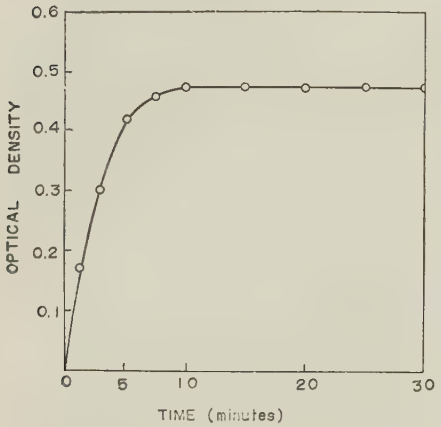

FIG. 1. Rate of color development of 3,7-dihydroxy-Δ⁵-cholenic acid treated with the H_2SO_4-acetic acid reagent.

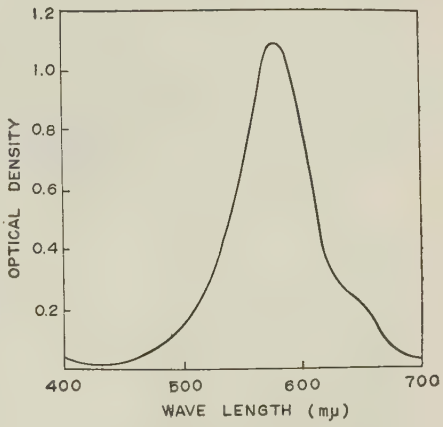

FIG. 2. Absorption spectrum of the chromophore obtained from 3,7-dihydroxy-Δ⁵-cholenic acid.

The calibration curve of 3,7-dihydroxy-Δ⁵-cholenic acid treated with the H_2SO_4-acetic acid reagent was found to obey Beer's law in the concentrations higher than 0.03 μmole of this cholenic acid (Fig. 3).

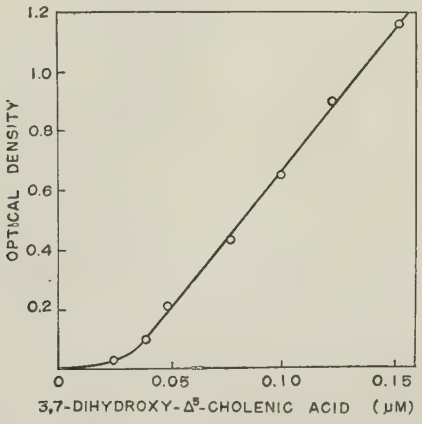

FIG. 3. Calibration curve of 3,7-dihydroxy-Δ⁵-cholenic acid treated with the H_2SO_4-acetic acid reagent.

As far as the linear portion of the calibration curve is used, sensitivity of this method is high enough to distinguish a change of optical density caused by less than 1 μg. of the acid ($\Delta E/\mu g. = 0.025$).

Specificity of This Reaction—Steroids and sterols, 31 in number, were tested with this

color reaction and the data were summarized in Table I. The development of a violet color was specific for 3,7-dihydroxy-Δ^5-steroids and the other steroids here tested gave almost no color or a faint yellow color, except that a pinc color was developed with some of the 3-hydroxy-Δ^4-steroids, which were obtainable from 3-keto-Δ^4-compounds by potassium borohydride reduction.

TABLE I

Specificity of the Lifschütz Reaction on Steroids

Steroid	Intensity of colorization (15 min. after the reagent is added)	Steroid	Intensity of colorization (15 min. after the reagent is added)
Cholanic acid	—	Methyl 3β-acetoxy-7-oxo-Δ^5-cholenate	—
Lithocholic acid	—	Desoxycorticosterone	—
Deoxycholic acid	—	Progesterone	—
Chenodeoxycholic acid	—	Coprostane	—
Cholic acid	—	Cholesterol	—
Taurocholic acid	—	7-Oxocholesterol	—
3-Oxocholanic acid	—	Cholestenone	+O
3,12-Dioxocholanic acid	—	7-Dehydrocholesterol	—
Dehydrocholic acid	—	7α(?)-Hydroxycholesterol	##V
3,12-Dihydroxy-7-oxo-cholanic acid	—	Scymnol	—
Δ^7-Cholenic acid	—	Reduced[1] desoxycorticosterone	+P
3β-Hydroxy-Δ^5-cholenic acid	—	Reduced[1] progesterone	+P
Apocholic acid	—	Reduced[1] methyl 3-oxo-Δ^4-cholenate	+V
Isodihydroxycholenic acid	+Y	Reduced[1] 3-oxo-Δ^4-cholenic acid	—
3,7-Dihydroxy-Δ^5-cholenic acid	##V	Reduced[1] cholestenone	±P
Methyl 3-oxo-Δ^4-cholenate	±Y		

O: orange; P: pinc; V: violet; Y: yellow.

1) Substances are reduced with KH_4B in methanol.

2. *Enzymatic Hydroxylation of 3β-Hydroxy-Δ^5-cholenic Acid*

Control Experiments—It was reported by Bergström and Wintersteiner (*10*) that cholesterol, methyl 3β-hydroxy-Δ^5-cholenate and related compounds were oxidized when heated in oxygen atmosphere at 80° for several hours giving their 7-oxo- or 7-hydroxy-compounds. Such an autoxidation of 3β-hydroxy-Δ^5-cholenate, however, was demonstrated only to a negligible extent under the conditions here adopted: Sodium 3β-hydroxy-Δ^5-cholenate was incubated at 37° for 2 hours with Tris* buffer (pH 7.4), TPNH and nicotinamide, no enzyme preparation being added. Some other experiments without

* The abbreviations here used are: ATP, adenosine-5'-triphosphate; DPNH, reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide; and Tris, tris(hydroxy-methyl) aminomethane.

enzyme preparation were carried out on the addition of heavy metal ions (Cu^{++}, Fe^{++}, Co^{++}) or human serum pretreated at 58° for 30 minutes. In neither of these experiments, any detectable amount of the oxidation product, *e. g.*, the supposed 3,7-dihydroxy-Δ⁵-cholenate was demonstrated.

Incubation Method—The liver was extirpated from rabbits weighing 1.5–2.5 kg., chilled in ice-water, and homogenized in a Potter-Elvehjem homogenizer with two volumes of 0.25 *M* sucrose solution. The homogenate was centrifuged for 10 minutes at 600×*g* and then at 44,000×*g* for 30 minutes. The resulting supernatant fluid, containing microsomal and soluble fractions of liver cells, was used, if not otherwise stated. With this enzyme preparation, 0.8 μmole of sodium 3β-hydroxy-Δ⁵-cholenate was incubated for two hours at 37° in the medium listed in Table II. Incubation was carried out in atmosphere of air in 50 ml-Erlenmeyer flask.

TABLE II

Incubation Medium

Enzyme preparation from the rabbit liver supernatant (44,000×*g*, 30 minutes) of 33% homogenate in 0.25 *M* sucrose.	30 ml.
Tris or phosphate buffer (pH 7.4)	200 μM
Sodium 3β-hydroxy-Δ⁵-cholenate	0.8 μM
Nicotinamide	50 μM
Cofactors	
TPN⁺, TPNH, or DPNH	0.5 μM
Glucose-6-phosphate or fructose-1,6-diphosphate	5.0 μM
Final volume	5–6 ml.
Conditions of the incubation: Gas phase, air; 37°, 2 hours.	

Extraction Method of Bile Acids—Five volumes of alcohol were added to one volume of the homogenate or of the incubation medium, and the resulting precipitates were filtered off and washed twice with small amount of alcohol. The extract was evaporated to drive off alcohol on the water bath, alkalinized by addition of 5 per cent sodium carbonate solution and extracted thoroughly with ether. The water phase was, then, acidified with dilute hydrochloric acid, and extracted twice with ether. The ether extract was washed twice with water, dried over sodium sulfate, evaporated to dryness, and subjected to the above-mentioned colorimetric determination.

Cofactor Requirement—It has been demonstrated that the enzymatic hydroxylation of the steroid molecules at the position of C-11 or C-21 by the adrenal enzyme systems required TPNH as a cofactor (*11*, *12*). The hydroxylation of the unsaturated bile acid here studied also was shown to require the same cofactor: When 0.8 μmole of 3β-hydroxy-Δ⁵-cholenate was incubated with the 44,000×*g* supernatant fluid of rabbit liver homogenate without any cofactor, none or a very small amount of 3,7-dihydroxy-Δ⁵-cholenic acid was detected. The addition of TPN^+ to the incubation medium apparently enhanced the formation of the dihydroxy acid, and furthermore the simultaneous addition of TPNH and glucose-6-phosphate accelerated the reaction remarkably as shown in Table III. DPNH with or without fructose-1,6-diphosphate, however, could not enhance the reaction.

Localization of Enzymatic Activity among the Cell Fractions—The samples of rabbit liver homogenate prepared with 0.25 *M* sucrose solution were separately centrifuged at 13,000×*g* for 10 minutes, 44,000×*g* for 30 minutes and 100,000×*g* for 2 hours, and each of these supernatant fluids and precipitates thus obtained was incubated with 3β-hydroxy-Δ⁵-cho-

lenate. In order to avoid the influence of TPNH-regenerating system, only TPNH was added as cofactor without glucose-6-phosphate. The 7-hydroxylating activity of rabbit liver was found to be localized in the 13,000×*g* or 44,000×*g* supernatant fraction. The

Table III

Cofactor Requirement

	Cofactor	3,7-Dihydroxy-Δ^5-cholenic acid formed (%)
Exp. 1.	None	1.1
	Glucose-6-phosphate	1.9
	TPN^+	2.3
	TPN^++glucose-6-phosphate	3.6
Exp. 2.	None	0.6
	TPNH	8.4
	TPNH+glucose-6-phosphate	20.9
	DPNH	0.0
	DPNH+fructose-1,6-diphosphate	0.1

activity could be separated into two components, *e. g.*, soluble and microsomal fractions, each of which was inactive when incubated separately, but fully active when incubated in combination (Table IV).

Table IV

Localization of Enzyme Activity among Cell Fractions

Cofactor added: TPNH (1.0 μM)

Enzyme preparation	3,7-Dihydroxy-Δ^5-cholenic acid formed (%)
13,000×*g* Supernatant	10.7
44,000×*g* Supernatant	13.2
100,000×*g* Supernatant (S)	1.2
'Heavy' microsomes (HMc)[1]	0.0
'Light' microsomes (LMc)[2]	0.0
HMc+S	8.5
LMc+S	10.2
HMc+LMc+S	13.4

1) Heavy microsomes: Microsomes precipitated at 13,000~44,000×*g*

2) Light microsomes: Microsomes precipitated at 44,000~100,000×*g*

Incubation Time—The influence of incubation time on the formation of the dihydroxy acid from 3β-hydroxy-Δ^5-cholenate is illustrated in Fig. 4. The incubation medium contains the 44,000×*g* supernatant fluid together with TPN^+ and glucose-6-phosphate. During

the first 60 minutes, 18 per cent of the substrate added was converted. After reaching a maximum of the conversion in 90 minutes (24 per cent converted), the formation of the dihydroxy acid was decreased.

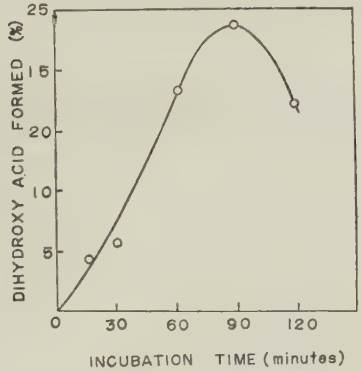

FIG. 4. Rate of the formation of 3,7-dihydroxy-Δ⁵-cholenic acid. Enzyme preparation: Supernatant fluid of 44,000 ×*g*; cofactors added: TPN⁺, glucose-6-phosphate.

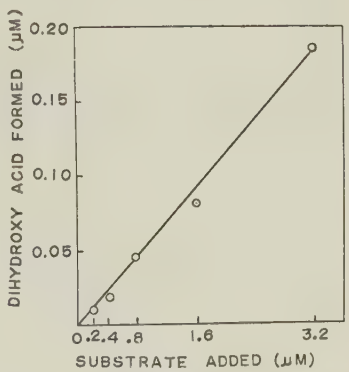

FIG. 5. Influence of substrate concentration upon the formation of 3,7-dihydroxy-Δ⁵-cholenic acid. Enzyme preparation: Supernatant fluid of 44,000 ×*g*; cofactors added: TPN⁺, glucose-6-phosphate; 37°, 2 hours.

Substrate Concentration—The effect of the substrate concentration on the hydroxylation reaction is illustrated in Fig. 5. The amount of 3,7-dihydroxy-Δ⁵-cholenic acid formed was proportional to that of the substrate added (between 0.2–3.2 μmoles), and a nearly equal portion of the substrate (6 per cent) was oxidized.

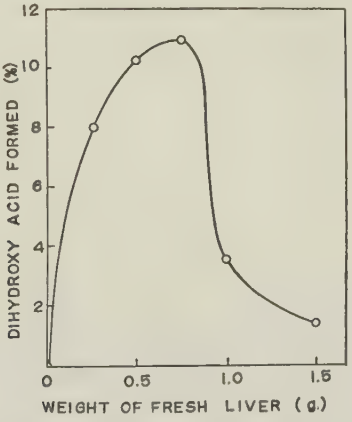

FIG. 6. Relationship between the formation of 3,7-dihydroxy-Δ⁵-cholenic acid and concentrations of enzyme preparation. Enzyme preparation: Supernatant fluid of 44,000×*g*; cofactors added: TPN⁺, glucose-6-phosphate; 37°, 2 hours. Numbers of the abscissa refer to the amount (*g.*) of fresh liver from which the supernatant fluid was prepared (see text).

Concentration of Enzyme Preparation—Experiments showed that as the amount of the enzyme preparation added was increased the formation of the dihydroxy acid was enhanced to reach a maximum, and then decreased (Fig. 6).

Excess of the enzyme preparation probably caused some further catabolism of the dihydroxy acid formed, say, its conversion into 3-keto bile acid or its conjugation. The concentration of the enzyme preparation that showed the maximum formation of the dihydroxy acid, however, was different sample by sample.

Influence of Some Drugs—Some drugs were tested as inhibitor, and results are shown in Table V. Cysteine, a protective reagent of sulfhydryl groups, inhibited the enzymatic

TABLE V

Influence of Some Drugs upon the Enzymic Activity

Enzyme preparation: Supernatant fluid of 44,000×*g*; cofactors added: TPN^+, glucose-6-phosphate.

Drug		3,7-Dihydroxy-Δ^5-cholenic acid formed (%)
None		1.7
Cysteine	30 μM	1.0
Iodoacetate	3.0 μM	5.9
$CuSO_4$	1.5 μM	1.6
$HgCl_2$	1.5 μM	2.8
EDTA	30 μM	12.9
KCN	50 μM	0.6

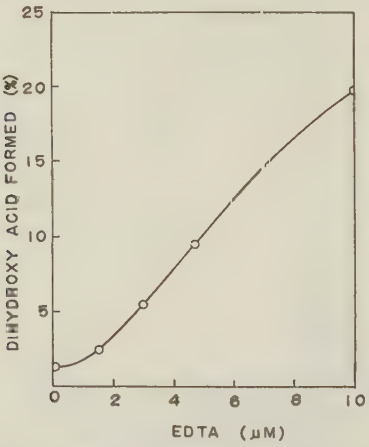

FIG. 7. Influence of EDTA upon the amount of 3,7-dihydroxy-Δ^5-choelnic acid formed. Enzyme preparation: Supernatant fluid of 44,000×*g*; cofactors added: TPN^+ glucose-6-phosphate.

activity considerably, while inhibitors of sulfhydryl group, iodoacetate, cupric sulfate and mercuric chloride, rather accelerated the reaction, more or less. Potassium cyanide de-

pressed the enzymatic reaction down to one third of the control. Addition of EDTA to the incubation medium strongly augmented the reaction, and as shown in Fig. 7, the formation of the dihydroxy-cholenic acid was found to be almost proportional to the concentrations of this drug.

DISCUSSION

It was reported by Bergström and Gloor (*13*) that an enzymatic system of rat liver homogenate can introduce a hydroxyl group into the C-7α position of deoxycholic acid. Their later experiments, however, showed that this reaction was not included in the proper metabolic pathway of cholesterol to the bile acid (*14*), and that the liver of rat, but not of rabbit, was capable of this reaction (*15*). The present experiment gave a strong evidence that when 3β-hydroxy-Δ⁵-cholenic acid was incubated with liver homogenate of rabbit and TPNH, it was hydroxylated to the postulated metabolite, 3,7-dihydroxy-Δ⁵-cholenic acid. The assumption that this metabolite is actually 3,7-dihydroxy-Δ⁵-cholenic acid is based mainly upon the color reaction specific for this dihydroxy acid (Table I). The paper chromatogram of this metabolite afforded an additional support for this assumption: The metabolite is was subjected to paper chromatography of Sjövall's system (*16*), and there appeared a spot (R_f=0.62) different from the starting material (R_f=0.86) as shown in Fig. 8. The R_f of this new spot apparently

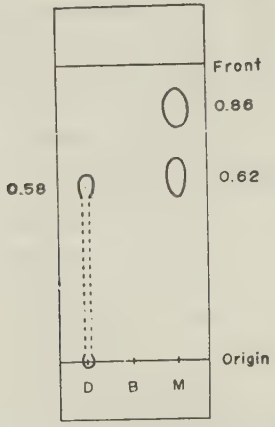

FIG. 8. Paper chromatogram of the metabolite of 3β-hydroxy-Δ⁵-cholenic acid. Sjövall's system (*15*). M: Extract from the incubation medium of 3β-hydroxy-Δ⁵-cholenate (Incubation: Supernatant fluid of 44,000×*g*, TPN^+, glucose-6-phosphate, 37°, 2 hours) B: Extract from the blank test; D: 3,7-Dihydroxy-Δ⁵-cholenic acid obtained by KH_4B reduction of 3β-hydroxy-7-oxo-Δ⁵-cholenic acid.

corresponds to that of a dihydroxy bile acid, but slightly different from the spot of 3,7-dihydroxy-Δ⁵-cholenic acid (m.p. 217°) obtained by potassium

borohydride reduction from 3β-hydroxy-7-oxo-Δ⁵-cholenic acid. This spot, therefore, might be that of the C-7-epimer of this dihydroxy acid or, less probably, of its further metabolized product.

Since the incubation of 3β-hydroxy-Δ⁵-cholenic acid was carried out in atmosphere of air, autoxidation of this acid should not be excluded (Bergström and Wintersteiner (*10*)), but, in fact, was found to be completely negligible under the conditions here adopted. Furthermore, requirements of TPNH and special components of liver cell fractions strongly suggest that the hydroxylation of this unsaturated bile acid was catalyzed by the enzyme system of the liver.

TPNH has been inferred as a cofactor for the enzymatic hydroxylation of steroids so far studied (*10*, *11*), and of a wide variety of drugs (*17*), and it might be required also as a cofactor for the conversion of squalene to lanosterol (*18*). Though not yet actually demonstrated, the description of ATP requirement for the hydroxylation of deoxycholic acid in rat liver homogenate (*13*) might suggest the requirement of TPNH. Furthermore Bergström and Gloor (*13*) reported that both microsomal and soluble fractions of rat liver cells were required for the hydroxylation reaction of deoxycholic acid. The present study likewise showed that the hydroxylation of 3β-hydroxy-Δ⁵-cholenate required TPNH and both of these fractions of rabbit liver, which, in turn, was reported to be incapable of hydroxylating deoxycholic acid (*14*). It is, therefore, highly probable that the hydroxylation reaction of rabbit liver preparation here shown is due to the very enzyme system that catalyzes the proposed 7-hydroxylation of cholesterol as the first step of the bile acid biosynthesis from this sterol. It is not certain that the very hydroxylase is different from the non-specific hydroxylase of drugs in the liver but since this hydroxylase is reported to require the microsomal fraction only, it is probably different from the hydroxylase system here shown with respect to the requirement of cell components.

In the sequence of the metabolic reactions of 3β-hydroxy-Δ⁵-cholenic acid, 3,7-dihydroxy-Δ⁵-cholenic acid once formed will be metabolized further, probably to 3-keto bile acid by 3β-hydroxysterol dehydrogenase (*8*, *6*) and, further, to the conjugated bile acid by liver microsomes (*19*). In fact, the present experiment showed that, in the early period of the incubation, 3,7-dihydroxy-Δ⁵-cholenic acid was formed from 3β-hydroxy-Δ⁵-cholenate, and in the later period or when the enzyme preparation was added in excess, partly disappeared (Figs. 4 and 6). Such disappearance of the metabolite formed could be due to the action of the above-mentioned enzyme systems, which contain in themselves sulfhydryl groups and require magnesium ions for the full reaction (*8*, *19*, *20*). This assumption might be confirmed by the findings that inhibitors of sulfhydryl group or EDTA markedly accelerated the hydroxylation of 3β-hydroxy-Δ⁵-cholenate to form 3,7-dihydroxycholenic acid.

SUMMARY

In order to study the hydroxylation mechanism of cholesterol, its analogue,

3β-hydroxy-Δ⁵-cholenic acid, was incubated with rabbit liver homogenate. The experimental results were as follows:

1. A method for the determination of 3,7-dihydroxy-Δ⁵-cholenic acid was described by use of the sulfuric acid-acetic acid reagent.

2. 3β-Hydroxy-Δ⁵-cholenic acid was converted to 3,7-dihydroxycholenic acid by incubation with 44,000×*g* supernatant fluid of rabbit liver homogenate. Both microsomal and soluble fractions were required for this reaction.

3. The hydroxylation reaction required TPNH as a cofactor, but not DPNH.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

VARIATION OF MACROMOLECULAR COMPONENTS OF PSEUDOMONAS-P AT DIFFERENT CULTURAL AGE

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(Received for publication, March 1, 1960)

As reported in the previous paper (*1*), the adaptability of *Pseudomonas-P* was varied during the growth phase. The capacity of induced formation of pyrocatecase was the the greatest in the cells harvested from the stationary phase of growth (O-cell). The cells of the earlier phase (Y-cell) had weaker adaptability than O-cells. Comparison of the free amino acids contents revealed that O-cells contained larger amounts of them than Y-cells.

This report will describe the differences between macromolecular components of Y- and O-cells as determined by electrophoretic analysis.

EXPERIMENTALS

Culture of the Organism—Preudomonas-P precultured for 24 hours on a peptone-bouillon agar slant was transferred into 150 ml. of peptone-bouillon medium, and incubated with shaking for 16 hours at 30°. A serial transfer of 1.2 ml. was made into 150 ml. of fresh medium. The culture was incubated under the same conditions as the first liquid culture. The cells harvested after the incubation period of 1 hour and 45 minutes were called Y-cells and the cells grown for 8 hours were called O-cells. Cell cultures were harvested by centrifugation, washed twice, and suspended in distilled water at concentration of 10 per cent (wet weight).

Preparation of Extracts—Cell suspensions (15 ml.) were sonicated in a 10 KC oscillator for 15 minutes. The temperature in the sonic chamber was maintained at about 3° by rapid circulation of ice cold water during the process. After the treatment, extracts were centrifuged at −5° at $20.000 \times g$ for 30 minutes. The yield of soluble materials from both O- and Y-cells was 95–96 per cent of the whole cells based on dry weight.

Fractionation of Cell-free Extract by Streptomycin—Ten per cent streptomycin solution (pH 7.4) was added dropwise to the whole extracts with gentle stirring. The pH of the solution was adjusted to 7.6 by 1/5 *M* tris solution. After the addition of streptomycin solution (approximately equal to 1/10 volume of the extract) gentle stirring was continued for 2 hours. The resultant white cloudy solution was centrifuged in the cold at $20,000 \times g$ for 10 minutes. The supernatant fraction was designated as S_1 fraction. The white precipitate was carefully dissolved in 1/3 *M* phosphate buffer (pH 7.6), and the clear solution was dialysed 100 volumes of 1/3 *M* phosphate buffer (pH 7.6). During the dialysis the outside buffer solution was stirred. After 2 hours, the dialysate was diluted to the original volume with distilled water. This preparation was designated as S_2.

Whole extracts, S_1 and S_2 fractions were dialysed overnight against tris-HCl buffer (μ=0.10, pH 8.35). These procedures were conducted in the cold room at 3°. The solutions were clarified by centrifugation at $20,000 \times g$ for 10 minutes prior to placing them

in the electrophoresis cells. Small amounts of resultant precipitate were discarded.

Electrophoretic Analysis—Electrophoretic determinations were made in a SPINCO-type-H-Tisselius electrophoresis assembly equipped with a schlieren optical system. The experiments were performed in an all-quartz cell assembly at 4°.

All the patterns shown in the text are photographs of the ascending limb of the cell after 60 minutes, under the current of 3 mA. in tris-HCl buffer (μ=0.10, pH 8.35).

Zone electrophoresis was carried out in a trough, 40×2×1 cm., packed with buffered starch at 5°. Tris-HCl buffer (μ=0.10, pH 8.35) was employed. After the run at potentials of 450-500 volts for 12-16 hours, the starch block was cut off in 1 cm. segments, and each segment was extracted with 3 ml. of water.

Contents of protein, RNA and polysaccharide were estimated by Lowry's method (*2*), orcinol reaction (*3*), and Roe's method (*4*), respectively.

RESULTS

Electrophoretic Patterns of Whole Extracts—In Fig. 1 a and b are the illustrative electrophoretic patterns of Y- and O-cells, showing that at least 7 components were present in the extracts. These components were designated according to their mobilities as the peak 1, 2, 3, 4, 5, 6 and 7.

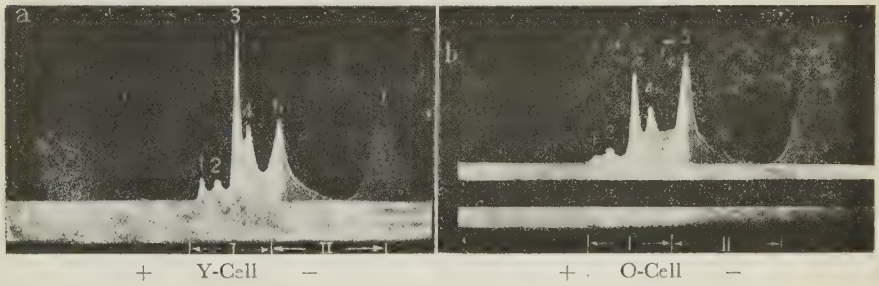


FIG. 1. Electrophoretic patterns of whole extracts.

Remarkable differences between Y- and O-cells were found in peaks 3 and 5. The amonut of substance in peak 3 of Y-cells was greater than that of O-cells. On the other hand, peak 5 is smaller in Y-cells than O-cells.

Samples taken from section I and II in Fig. 1 b were analysed by spectrophotometry. In the ultraviolet region from 230 to 300 mμ, the absorption spectrum of both sections gave similar patterns both qualitatively and quantitatively, indicating the contents of RNA were the same in both sections. In the visible region from 390 to 550 mμ, a significant difference was observed (Fig. 2 a and b). The sample from section II absorbed at 420 mμ, indicating the presence of cytochroms and the other section did not show appreciable absorption.

These results suggested that the peak 3 was a nucleic acid rich component and the peak 5 was a cytochrome containing component.

Zone Electrophoretic Separation of Whole Extracts—The whole extract of cells was separated by zone electrophoresis as shown in Fig. 3.

Four distinct protein peaks (1, 2, 3, 4) and two RNA peaks (I, II) were

observed. The RNA of peak I was free RNA and the RNA of peak II was

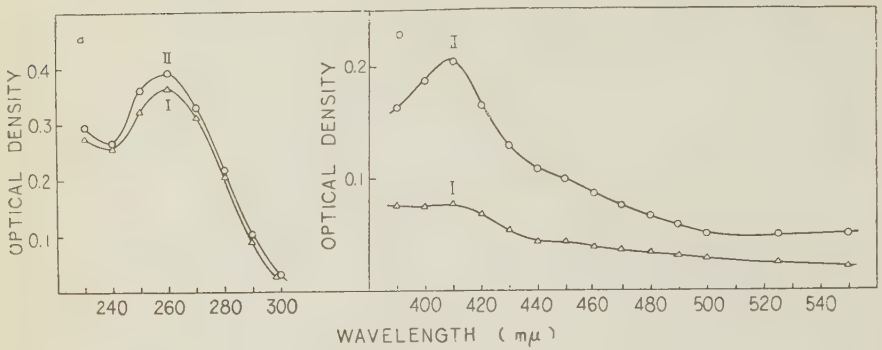


Fig. 2. Spectrophotometry of the variable components.

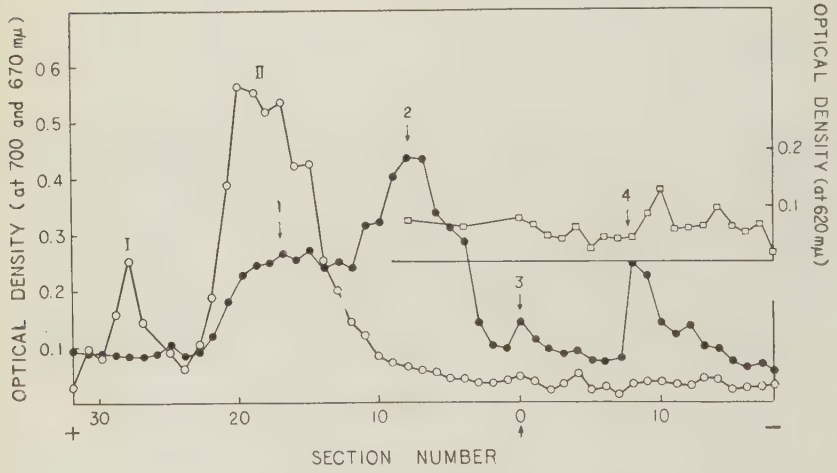


Fig. 3. Zone electrophoresis of the whole extract of O-cells.
—●—: protein (700 mμ), —○—: RNA (670 mμ),
—□—: polysaccharide (620 mμ).

accompanied by protein. Therefore peak II was considered as a ribonucleoprotein. Polysaccharide was found in the region of peak 3 and 4.

Comparing these results with the free electrophoretic patterns, it was concluded that the peaks 2, 3, 5 and 7 in the free electrophoretic pattern were respectively RNA, ribonucleoprotein, cytochrome rich fraction and protein attached with polysaccharide. The peak 1 was thought to be free DNA judging from its mobility.

Cytochrome Contents of Y- and O-Cells—Fig. 4 shows the absorption spectrum of the whole extracts of O- and Y-cells in the visible region from 500 to 600 mμ. O-cells contained 6 to 7 times as much cytocerome as Y-cells on a dry weight basis of the extracts.

Fractionation of the Whole Extract by Streptomycin—It is known that streptomycin precipitates nucleic acid components of biological fluids (*5*, *6*, *7*,). Nuclecic acid precipitation in these bacteria was greatly influenced by the amounts of phosphate ion present. It was found that in the extracts prepared with 1/10 *M* phosphate buffer no precipitation occurned, and in the

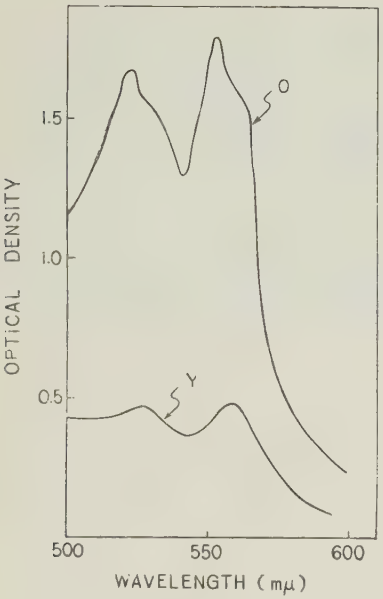
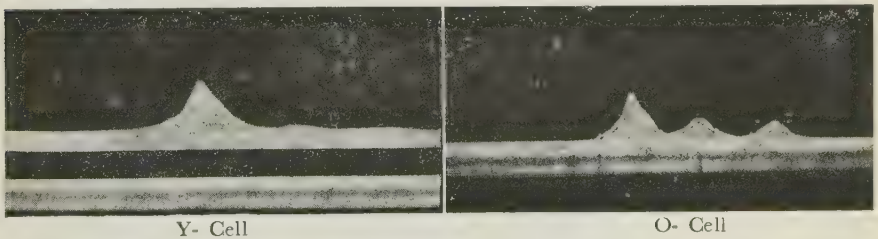


Fig. 4. Absorption spectra of the whole extracts of O- and Y-cells.

Fig. 5. Absorption spectra of the S_1 and S_2 fractions.
—△—: S_1, —○—: S_2.

phosphate free extracts (tris buffer extracts), a large amount of cloudy precipitate was formed by addition of streptomycin. The supernatant fraction (S_1) and the streptomycin precipitate fraction (S_2) were obtained as described in the previous section.



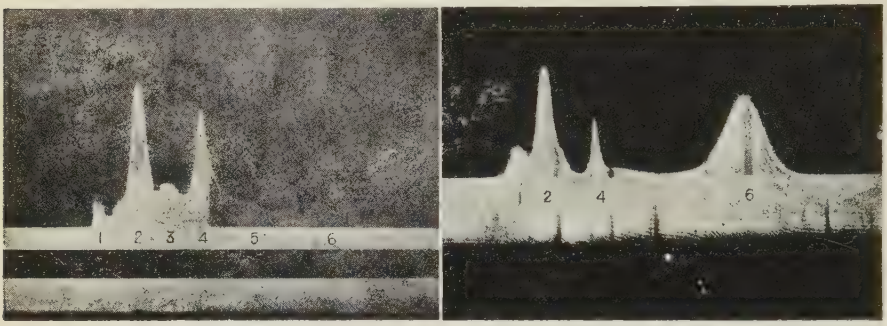
Y- Cell O- Cell

Fig. 6. Electrophoretic patterns of the S_1 fraction of O- and Y- cells.

Spectrophotometric measurements showed that the ratio of absorption at 280 mμ to that at 260 mμ was 1.15 in the S_1 fraction and 0.54 in the S_2 fraction (Fig. 5). This indicated that the S_1 fraction was almost free from

nucleic acid and all of the nucleic acid came to the S_2 fraction.

Examination of S_1 and S_2 Fractions by Their Free Electrophoretic Patterns—The S_1 fraction was composed of three main components, and as show in Fig. 6, the amount of each component in O-cells was higher concentration than the corresponding componet in Y-cells.



Y- Cell C- Cell

FIG. 7. Electrophoretic patterns of the S_2 fraction of O- and Y- cells.

Fig. 7 shows the electrophoretic patterns of the S_2 fractions. The S_2 fraction obtained from Y-cells was composed of six distinct peaks. The amounts of peaks 2 and 4 in Y-cells were greater than the corresponding peaks in O-cells. Peak 3 was found only in Y-cells.

DISCUSSION

Comparison of the electrophoretic patterns are valid only when the extracts are prepared under the strictly controlled conditions. All of the cell-free extracts used in these experiments were prepared identically as specified in the above description of the methods. The yields of cell-free extracts were always 95–96 per cent on dry weight basis and several extracts prepared under these conditions were found to give the reproducible patterns.

As shown in Fig. 1, the electrophoretic patterns of extracts obtained from Y- and O-cells showed significant differences in the relative amounts in several components. In Y-cells (log-phase cells), the ribonucleoprotein content was higher than in O-cells (stationary-phase cells). On the other hand, the amount of cytochrome fraction was larger in O-cells.

As shown in Fig. 6, the cytochrome content of the cells showed a very significant difference depending on their cultural age. From further investigations of this point, it was found that the content of cytochrome of the cells increased parallel to the growth of the cells (*8*). It was considerd, therefore, that the composition of the cellular components was continuously changing during the multiplication of the cells. This may be a result of the change in the nutritional condition which causes controlled alteration of cellular metabolism during the increase of cell population.

SUMMARY

1. Electrophoretic analysis of the sonic extracts of *Pseudomonas-P* revealed that they were composed of at least 7 componets.

2. Each component was separated by zone electrophoresis and characterized by chemical analysis and spectrophotometry.

3. These components were: free DNA, RNA, ribonucleoprotein, cytochrome rich fraction and protein accompanied with polysaccharide.

4. It was found that the content of ribonucleoprotein was higher in the early log-phase cells than in the stationary-phase cells. On the other hand, the amount of the cytochrome rich fraction was greater than the stationay-phase cell.

5. Streptomycin precipitated all of the nucleic acids containing fractions under the specified condition.

6. The variation of cellular components was discussed from the physiological view point.

The authers wish to express their gratitude to Dr. B. Maruo and Dr. H. Takahashi for their valuable suggestions and critisism during the course of this work.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

INCORPORATION OF AMINO ACIDS INTO PROTEIN OF PSEUDOMONAS CELL FRACTIONS

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(Received for publication, March 2, 1960)

It was observed that the contents of some of the macromolecule containing-components in sonic extracts of *Pseudomonas P* (*1*), varied during its growth cycle. These components were a ribonucleic acid-containing fraction and the other was a cytochrome-containing one. They were mainly associated with the particulate fractions of this microorganism. We studied about the function of these components in protein synthesis.

Before investigating the capacity of these fractions in protein synthesis *in vitro*, it is of some interest to examine their behaviour in protein synthesis in the intact cells. Some data on this subject have been reported with *Bacillus megatherium* (*2*), *Pseudomonas fluorescens**, *Escherichia coli* (*3*), *Azotobacter vinelandii* (*4*), and with yeast (*5*).

EXPERIMENTALS

Culture of the Microorganism—A precise description was given in the previous report (*1*). In this experiment, cells were grown in pepton-bouillon medium, then transferred into fresh medium and incubated for 3 hours and 40 minutes. These cells were collected by centrifugation, washed twice and resuspended in deionized water at the concentration of 10 per cent on a wet weight basis.

Preparation of the fractions: *1. Sonic Disintegration*—The cell suspension was disintegrated for 10 minutes in a 9 KC oscillater (Toyo Riko 505), at 100 watt out put, and centrifuged at $4,000 \times g$ for 15 minutes. The supernatant solution (S_1) was centrifuged at $20,000 \times g$ for 1 hour, and the precipitate (LP) was collected. The $20,000 \times g$ supernatant solution (S_2) was centrifuged in the cold at $105,000 \times g$ for 1 hour. The sediment and supernatant fractions were designated as SP and S fraction respectively (Scheme 1).

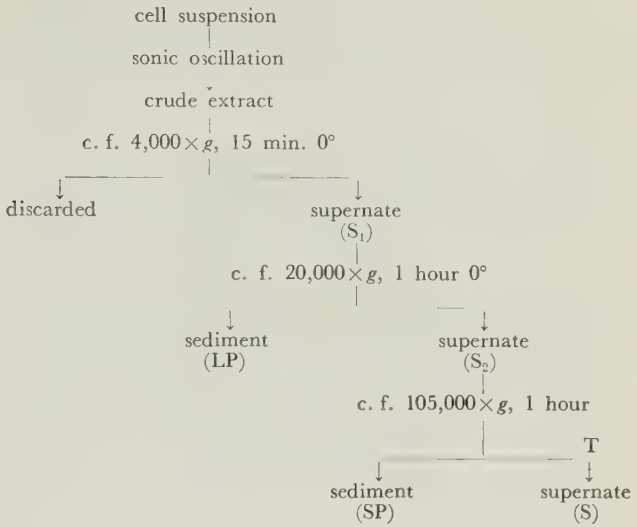
2. Lysozyme Treatment—Pseudomonad cells were converted to protoplasts by lysozyme treatment in supporting medium, and the protoplasts were easily disrupted by dilution. The method used is described in detail. A half ml. of cell suspension was incubated with 8.5 μM of EDTA, 300 μg. of crystalline lysozyme, 75 μM of tris-HCl buffer (pH 8.0) and 150 μM of KCl in a total volume of 3 ml. The incubation was run at room temperature for 10 minutes. After incubation the protoplasts were centrifuged in the cold, treated with 0.5 ml. of DNase solution (100 μg./ml.), and were brought to 2.5 ml. with distilled water. Disrupted protoplasts were fractionated into sediment and supernatant

* personal communications

fractions by centrifugation at 0° and 20,000×*g* for 20 minutes. These fractions corresponded to cytoplasmic membrane and its supernate.

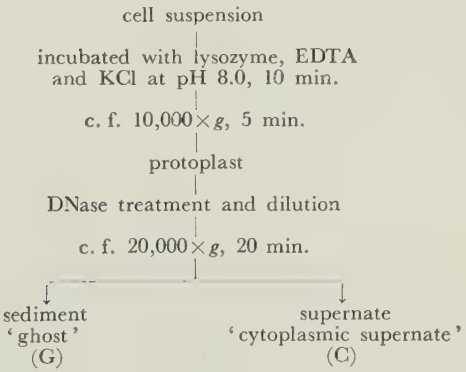
SCHEME 1

Preparation of Sonic Extract



SCHEME 2

Procedure for Lysozyme Treatment



Electrophoresis—Electrophoresis in free solution or on starch were run by the same methods as described in the previous report (*1*).

Incorporation Experiments—Fifty mg. of cells were suspended in 8.8 ml. of *M*/10 phosphate buffer (pH 7.3) and 1.0 ml. of 10 per cent casamino acid (DIFCO) and incubated aerobically at 30°. After the temperature equilibrium, 0.2 ml. of C^{14}-labeled algal protein acid hydrolysate was added and the suspension was incubated for an appropriate time. Each fraction was treated according to a modified Schneider's method. Protein and RNA were determined by means of the phenol-reagent and orcinol method respectively.

Radio activity of the samples was determined with an end-window Geiger-Müller counter. Coincidence and self-absorption corrections were found unnecessary with the levels of radioactivity and sample size used.

RESULTS

Properties of Cell Fractions Obtained From Sonic Extract—The initial extract prior to high speed centrifugation (S_2) was analysed with the Spinco Model E ultracentrifuge. The ultracentrifuge pattern is shown in Fig. 1.

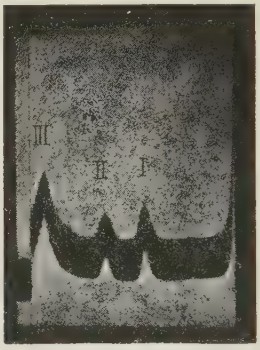


FIG. 1. Ultracentrifuge pattern of S_2 fraction.
Photograph was taken after the sedimentation for about 20 minutes at 20° and 39,460 r.p.m.

Peak I corresponds to the SP fraction. Peak II and peak III are the main components of the S fraction.

Table I summarizes the distribution of protein and ribonucleic acid in the various fractions. The LP fraction was low in RNA content and contained much lipid material.

TABLE I

Distribution of RNA and Protein in Fractions

Fraction	per cent of total	
	RNA	Protein
LP	2.1	6.0
SP	28.1	16.0
S	69.6	78.0

SP was the most RNA rich fraction of the three, and cytochrome was mainely associated with this fraction. The S fraction was associated with more than 70 per cent of the RNA and protein content of the whole extract.

In Fig. 2. a, b and c are the boundary electrophoretic patterns of LP, SP and S respectively.

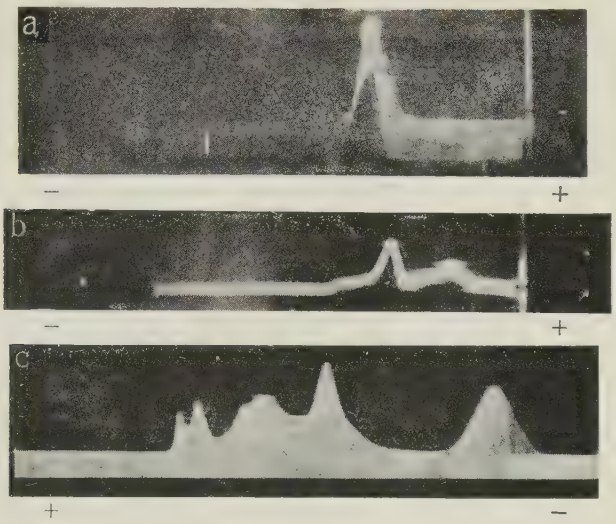


Fig. 2. Boundary electrophoretic patterns of LP, SP and S.
(Ascending limb, tris-HCl buffer, ionic strength 0.01, pH 8.35)

LP showed a single peak, SP was composed of three main components and in S at least six components were observed.

In order to know the characteristics of each component of the fractions, zone electrophreses of the SP and S fractions were carried out. As shown in Fig. 3, the SP fraction was separated into three components (a). Peak I

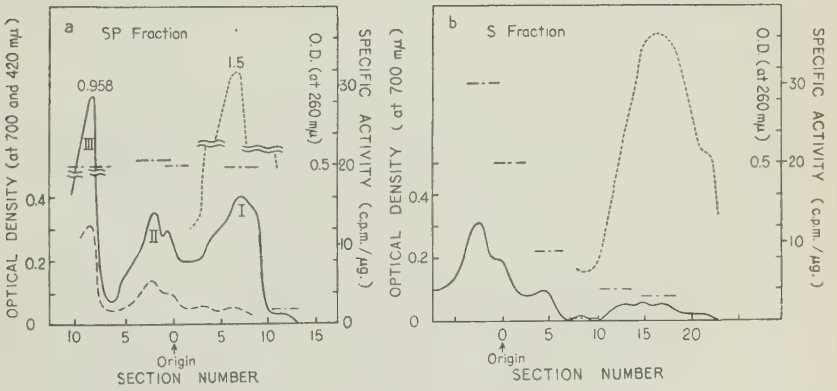


Fig. 3. Zone electrophoretic patterns of SP and S.
a. 250 Volt, 4.4 mA. 13.5 hr. b. 200 Volt, 4 mA. for 14 hr.
····· 260 mμ. – – – – 420 mμ. ——— 700 mμ. —·— specific activity.

was called ribonucleoprotein because of the coincidence of ribonucleic acid

with about the same amount of protein and Peak II and III, which showed absorption at 420 mμ, were cytochrome containing components. Fig. 3 also shows the zone electrophoretic pattern of the S fraction (b). The fraction was separated into a protein component and a ribonucleic acid rich fraction.

Incorporation of C^{14}*-Amino Acids*—Fig. 4 shows the time course of C^{14}-amino acid incorporation into the protein of intact cells.

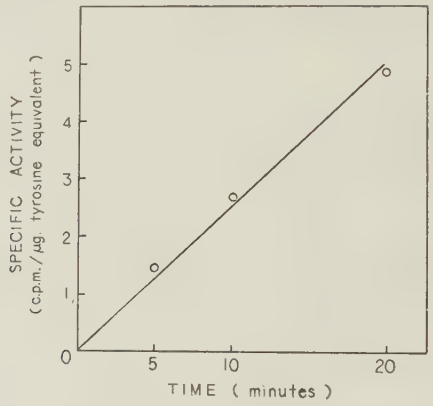


FIG. 4. Time course of amino acid incorporation *in vivo*.

Five ml. of cell suspension was incubated with C^{14}-amino acid (25,000 c.p.m.) as described in the method.

The reaction proceeded linearly for at least 20 minutes.

It would be expected that the site of protein synthesis would have a high specific activity when the reaction was restricted to a short period. Therefore, two minutes after the addition of the C^{14}-amino acid mixture,

TABLE II

Incorporation of Amino Acids into Protein of Pseudomonad Cell Fractions in vivo

Fraction	total protein (μg. of tyrosine) equivalent)	total activity (c.p.m.)	specific activity (c.p.m./μg. tyr.)
LP	610	15,800	26
SP	440	9,700	23
S	6,120	26,100	4.3

Five ml. of cell suspension was incubated with C^{14}-amino acid mixture (500,000 c.p.m.) in *M*/15 phosphate buffer at 30° for 2 minutes.

the reaction was stopped with sodium cyanide, and the cells were separated from the reaction medium by centrifugation at 0°. They were washed once

with water and disrupted by sonic oscillation. The sonic extract was separated into LP, SP and S fractions by centrifugation as described in the previous section.

As shown in Table II, the radioactivity of the soluble fraction was nearly equal to the sum of the radioactivity of the particulate fractions. However, the specific activity of the particulate fraction was higher than that of the soluble fraction. The SP and S fractions were composed of several components and they could be separated by zone electrophoresis. After the separation by the starch electrophoresis, the radioactivity of each component in SP and S fractions was estimated.

As shown in Fig. 3. a and b, the three components of the SP fraction showed nearly equal specific activities. Evaluation of the labeling in the components of the S fraction, however, showed an appreciable difference in the specific activities of the three components. It could be considered, therefore, that the particulate fraction was the site of protein synthesis and the proteins of the soluble fraction was synthesized at a different rate from the particulate fractions.

TABLE III

Incorporation of C^{14}-Amino Acids in Isolated Fractions

	LP	LP+S[1)]	SP	SP+S[1)]	S
radioactivity (c.p.m./100 μg. tyrosine)	65	27	42	5.0	4.0

reaction condition: 30° aerobic for 60 min.

reaction mixture: 0.5 *M* phosphate buffer (pH 7.4,) 0.2 ml.
10 per cent casamino acid 0.1 ml.
180,000 c.p.m./ml. C^{14}-amino acid mixture 0.2 ml.
0.5 mg. protein for SP and 1.1 mg. protein for S. total 1.8 ml.

1) Particulate fraction was separated from soluble fraction after the incubation and the specific activities were estimated as to LP or SP only.

Table III shows the results of the experiment with isolated fractions. The abilities of the LP and SP fractions to incorporate C^{14}-amino acids were much higher than that of S. S showed a marked inhibitory effect on the incorporation of C^{14}-amino acids by particles.

The activities of the particles *in vitro* were very weak compared to that *in vivo*. Therefore, it was thought that sonic disintegration of cells was not suitable for further investigation to clarify the function in protein synthesis. Thus, lysozyme treatment was employed as a milder method for disruption of cells.

Disrupted protoplasts were separated into a so-called 'Ghost' fraction (G), and a cytoplasmic supernate (C), by centrifugation. An electronmicrograph of the 'Ghost' fraction showed that this was composed of cytoplasmic membranes with granules attached. After sonic disintegration of this

'Ghost' fraction, the cytoplasmic membrane could be fractionated into particulate fractions, LP and SP.

TABLE IV

Distribution of RNA and Protein in Fractions of Lysozyme Treated Cells

per cent of total

fraction	RNA	protein
G	42.5	51.8
C	57.5	48.2

Protein and ribonucleic acid were distributed in both fractions, G and C, in nearly equal amounts.

Cells labeled *in vivo* were treated with lysozyme and fractionated into the G and C fractions. The specific activities of each fraction increased linearly during the first 30 minutes of the incubation period, and the rate of incorporation of amino acids into each fraction was nearly equal. These results shown in Fig. 5. suggested that some interaction between G and C fractions existed in the protein synthesis.

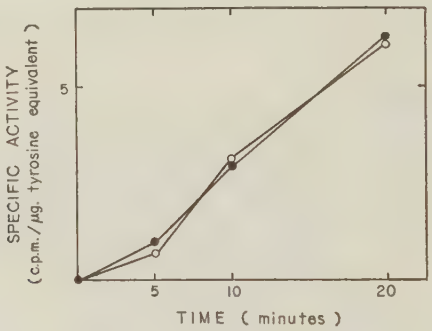


FIG. 5. Amino acid incorporation into the G and C fractions *in vivo*.

Conditions of incorporation was described in the experimental section. —●— G fraction, —○— C fraction.

In order to clarify this point further investigations were carried out with disrupted protoplasts. The results will be reported elsewhere (*6*).

DISCUSSION

It was found that the rate of incorporation of labeled amino acids into proteins of large particles (LP) and small particles (SP) *in vivo* was much faster than incorporation into the supernatant fraction (S). Isolated fractions obtained from sonic extract, however, had very weak activities to incorporate amino acids, and the ratio of the activity of LP, SP and S was 16 : 11 : 1.

Disrupted protoplasts of *Bacillus megatherium* (*2*), containing cytoplasmic membrane incorporated amino acids very actively, and further investigation with isolated fractions obtained from disrupted protoplasts showed that the cytoplasmic membrane fraction incorporated amino acids very actively, and the cytoplasmic supernate could not incorporate amino acids without the aid of the membrane fraction.

The membrane fraction gave particles, LP and SP, by sonic disintegration, but these isolated particles did not show appreciable activities.

In *Azotobacter vinelandii*, the particulate fraction had only a limited capacity to incorporate amino acids into protein in comparison to the activity exhibited by particles associated with the cell membrane.

It was concluded that the cytoplasmic membrane fraction was a main site of protein synthesis, and cytoplasmic protein was synthesized in a manner utilizing the cytoplasmic membrane. Our studies confirmed further these conclusions, and suggested that the LP and SP particles adhering to the cytoplasmic membrane had a very important role in protein synthesis of this microorganism.

SUMMARY

1. Cell fractions were prepared from *Pseudomonas-P* by sonic disintegration and differential centrifugation.

2. These fractions were composed of large particles, sedimentable at low speed centrifugation ($20{,}000 \times g$), small particles, sedimented at $105{,}000 \times g$, and the soluble fraction.

3. Chemical, ultracentrifugal and electrophoretic analysis gave some characteristics of each fraction.

4. Both *in vivo* and *in vitro* experiments showed that the rate of amino acid incorporation into the particulate fractions was much higher than in the soluble fraction.

5. The activity of the particulate fraction was markedly inhibited by the soluble fraction.

6. A 'Ghost' fraction and a cytoplasmic supernate were obtained by lysozyme treatment in hypertonic medium and subsequent osmotic shock of the protoplasts.

7. These two fractions incorporated amino acid at the same rate *in vivo*.

8. The role of the particulate fraction of *Pseudomonad* cells was discussed.

The authors wish to express their many thanks to Prof. B. Maruo and Dr. H. Takahashi for their kind guidance and many useful suggestions during the course of this work.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

AMINO ACID INCORPORATION BY A BACTERIAL CELL-FREE SYSTEM

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(Received for publication March 10, 1960)

During the last few years, many workers (*1*~*5*) obtained bacterial cell-free systems which incorporate amino acids into the protein fractions. It appears that the amino acid incorporating activity was mainly associated with the particulate fractions (*4*), or cell mambrane fragments (*1*, *2*, *3*, *5*).

Separation and identification of macromolecular component within the cell of *Pseudomonas-P* were investigated in this laboratory. During the course of the experiment, it was suggested that activity of protein synthesis was associated with the particulate fractions or cell membrane of this micro-organism.

When this bacterium was disrupted by sonic oscillation, such a preparation showed only a little activity of amino acid incorporation. A cell-free preparation which was obtained by means of osmotic shock of the protoplast incorporated C^{14}-labeled amino acids with an activity comparable to the intact cells.

The properties of the preparation and the conditions which were essential for the amino acid incorporation in this system will be described.

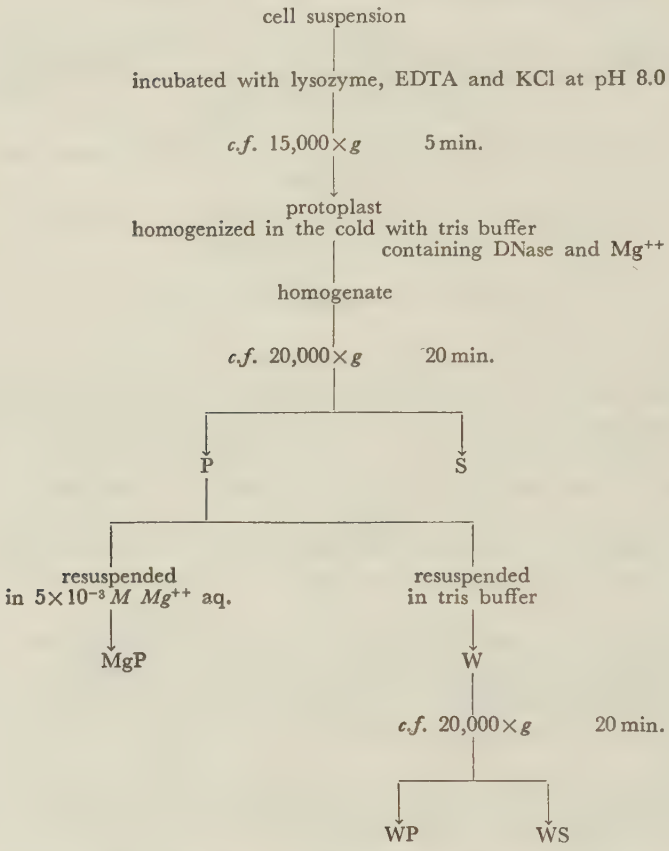
EXPERIMENTAL

Microorganism—Pseudomonas-P (*6*), was grown in the pepton-bouillon medium for 16 hours at 30° under aerobic conditions. From this culture 1.2 ml. were inoculated into 150 ml. of a fresh medium and incubated under the same conditions. After 3 hours and 40 minutes, logarithmically growing cells were harvested, washed twice with deionized water, and resuspended in deionized water at a concentration of 10 per cent on wet weight basis.

Preparation of Protoplast and Homogenate—The incubation mixture contained one ml. of cell suspension, 600 μg. of crystalline lysozyme (N.B.C.), 17 μM of EDTA, 3 mM of KCl and 3 ml. of tris buffer (*M*/20, pH 8.0). Final volume was 6 ml. This mixture was incubated at 30° for 30 minutes. By this treatment cells became protoplasts which could easily be disrupted by osmotic shock. Protoplasts thus obtained and collected by centrifugation, were homogenized by rapid agitation with a pipette by adding 0.2 ml. of DNase (100 μg./ml.) dissolved in $5\times10^{-2}M$ $MgSO_4$ solution (pH 7.4). This was gradually diluted with tris buffer (pH 7.4) to 2 ml.

SCHEME 1

Procedure for Preparation of the Cell-Free System



The homogenate was fractionated to precipitate (P) and supernatant (S) fractions by centrifugation at 20,000×*g* for 20 minutes. The P fraction resuspended in $MgSO_4$ solution of $5\times10^{-3}M$ and that resuspended in $M/20$ tris buffer (pH 7.2) were designated as MgP and W respectively. Subsequent centrifugation of the W fraction gave precipitate (WP) and supernatant (WS) fractions.

Incorporation—The reaction was carried out at 30° with shaking, and stopped by adding an equal volume of 10 per cent perchloric acid or 1/10 volume of $10^{-1}M$ sodium cyanide and chilled in the ice bath. The reaction mixtures contained intact cells or subcellular fractions, phosphate buffer (pH 7.2), casamino acid (DIFCO) and mixture of radioactive amino acids. Details will be described in the legends.

Measurement of Radioactivity—The protein fraction prepared by a modified Schneider's method (*7*), was dissolved in dilute aqueous ammonium hydroxide solution. An aliquot was put on the planchette and radioactivity was measured with an end-window Geiger-Müller counter. The weight of a sample was always less than 0.2 mg./cm², and the correction for self-absorption was not carried out.

Analysis—Protein content was estimated by Lowry's metnod (*8*), and expressed as

tyrosine equivalents. Ribonucleic acid was separated by Schmidt and Thannhauser's method (*9*) and estimated by orcinol reaction and ultraviolet absorption at 260 mμ.

C^{14}-Amino Acid Mixture—Acid hydrolysate of uniformly labeled C^{14}-algal protein was used (*10*).

RESULTS

Amino Acid Incorporation by Intact Cells and Homogenate—Amino acid incorporation by intact cells proceeded linearly for 30 minutes, and this continued for 30 minutes more.

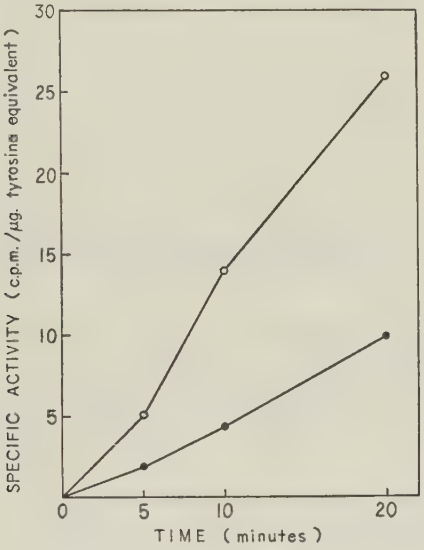


FIG. 1. Amino acid incorporation by intact cells and homogenate.

Incubation mixtures contained 2 mg. of casamino acid, C^{14}-amino acid mixture (38,000 c.p.m.), 1.2 ml. of *M*/5 phosphate buffer and 0.5 ml. of cell suspension or homogenate. They were incubated as mentioned in the previous section. —○— Intact cells, —●— homogenate.

Under the same conditions homogenate incorporated amino acids at the rate of 1/3 of that of the intact cells. (Fig. 1.)

After the reaction was stopped by sodium cyanide, homogenate was separated by centrifugation to supernate and precipitate. Both fractions were labeled as shown in Fig. 2 a.

The viable cell count of this homogenate was less than 0.01 per cent of original cell suspension, and no intact cell was detected by electron microscopic observations.

Amino Acid Incorporation by Isolated Components—The S fraction or the P

fraction resuspended in buffer (W) did not incorporate amino acids into the protein fraction by itself. (Fig. 2. b)

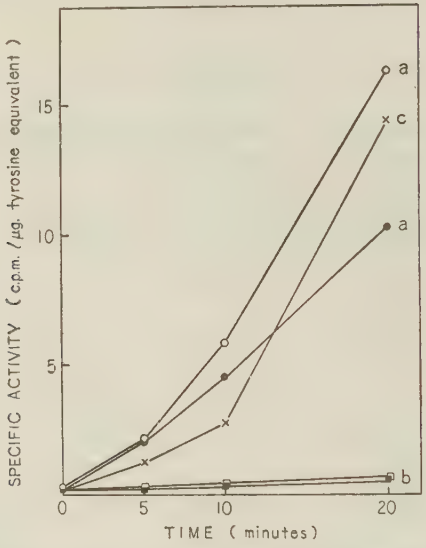


Fig. 2. Amino acid incorporation by isolated components.

(a) The homogenate was incubated with C^{14}-amino acid mixture and separated by subsequent centrifugation to precipitate and supernate. —●— Precipitate, —○— supernate.

(b) The W fraction and the S fraction was respectively incubated with C^{14}-amino acid mixture. —□— W, —■— S.

(c) The P fraction resuspended in the S fraction was incubated with C^{14}-amino acid mixture. —×—

Each flask contained 1 ml. of subcellular fraction, 1 mg. of casamino acids, C^{14}-amino acid mixture (40,000 c.p.m.) and 2.4 ml. of *M*/5 phosphate buffer. The final volume was 4.0 ml.

Though the addition of the S fraction to the W did not restore the activity, the P fraction resuspended in the S fraction showed nearly the same activity as that of the P fraction in homogenate (Fig. 2 c).

From these results, it was expected that the composition of the resuspending medium was important for the activity of the P fraction.

It was found that the magnesium ion was essential as a component of resuspending medium.

The P fraction resuspended in magnesium solution of appropriate concentration completely restored its activity, but the addition of magnesium ion to the W did not restore its activity.

TABLE I

Effect of Resuspending Medium on Activity of P Fraction

Resuspending Medium	Addition	Specific Activity[1] (c.p.m./μg tyrosine equivalent)
M/20 tris buffer (pH 7.2)	—	1.4
,,	heated[2] S fraction	1.9
Heated S fraction	—	33
$MgSO_4$ (4×10^{-3} *M*)	—	29
,,	heated S fraction	31

1) Preparations were incubated with C^{14}-amino acid for 30 minutes.

2) S fraction was treated in boiling bath for 10 minutes and denatured protein was removed by centrifugation. One ml. was added to the incorporation mixture.

All the conditions were the same as mentioned in legned of Fig. 2 except for the amount of C^{14}-amino acid mixture (150,000 c.p.m.).

Effect of Magnesium Ion on the Incorporation System: *1. Magnesium Ion and Activity*—Suspensions of precipitate (MgP) were prepared in various concentra-

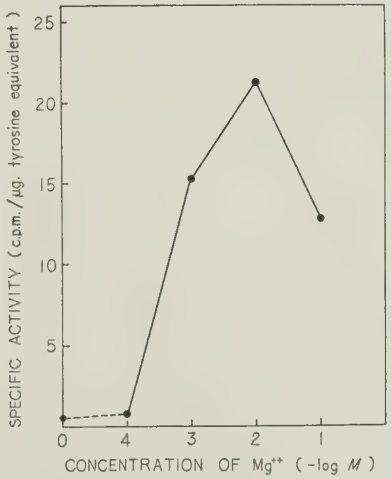


FIG. 3. Effect of concentration of magnesium ion in resuspending medium on the activity.

Each flask contained 1 ml. of preparation, resuspended in various concentrations of $MgSO_4$, 1 mg. of casamino acid, C^{14}-amino acid mixture (60,000 c.p.m.), 0.9 ml. of *M*/5 phosphate buffer (pH 7.2). Final volume was 4.0 ml. Incubation was continued for 30 minutes.

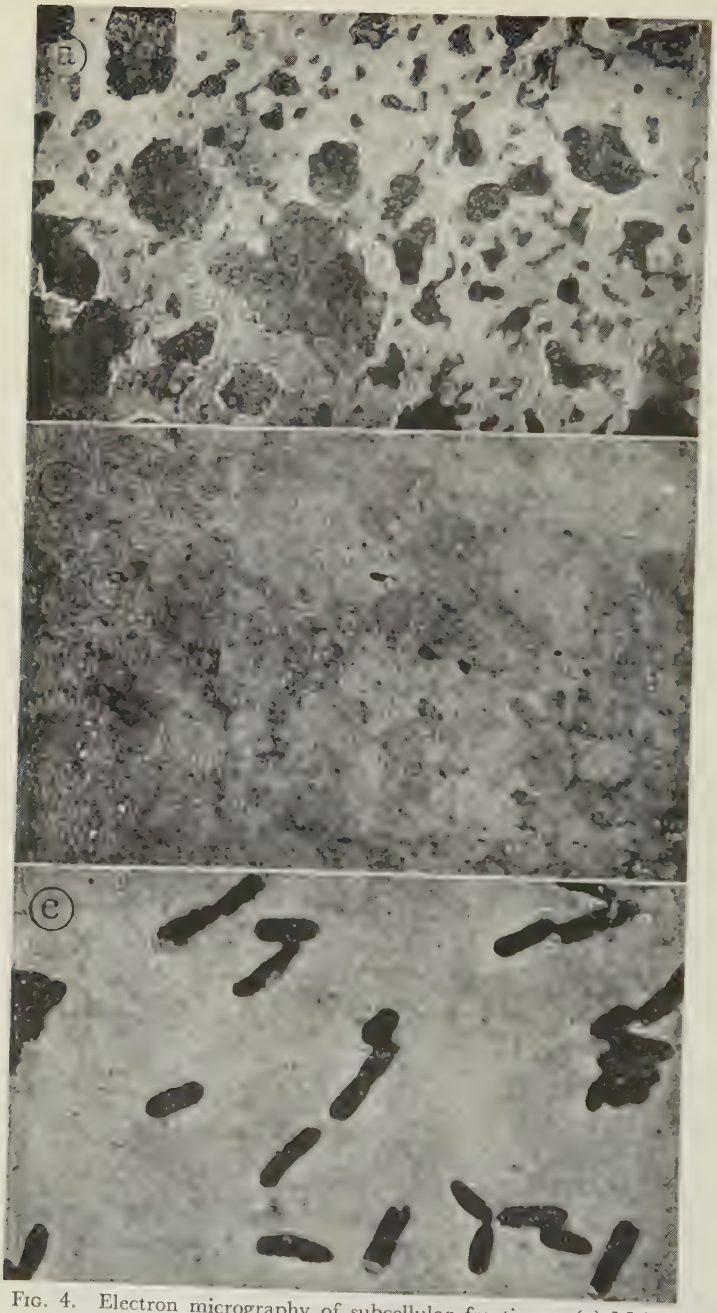


FIG. 4. Electron micrography of subcellular fractions. (a) MgP fraction, ×5750, (b) WP fraction, ×5750, (c) Intact cells of *Pseudomonas P*, ×5750.

tion of $MgSO_4$, and each preparation was examined as to its activity under the same conditions.

Optimal concentration of $MgSO_4$ was $10^{-2}\,M$. At concentrations less than $10^{-4}\,M$ more than 95 per cent of maximal activity was lost.

Again, addition of magnesium ion to the preparation low in magnesium concentration had no effect.

2. Release of Macromolecular Components from the P Fraction in Low Concentration of $MgSO_4$—Comparing to the MgP fraction which was active in amino

TABLE II

Analysis of Released Components

Component \ Fractions	MgP	WP	WS
Protein[1] (μg. tyrosine equiv.)	220	85	130
RNA[1] (μg.)	770	170	600
Particles[2]	—	—	56s, 46s, — 31s
Amino Acid activating enzymes[3] (POP μM exchanged/10 min.)	0.75	0.06	0.90

1) One ml. of each fraction was analysed according to the methods described in the previous section.

2) Sedimentation analysis was carried out with Spinco ultracentrifuge model E.

3) Each flask contained 0.1 ml. of preparation, 1 mg. of casamino acid, 5 μM of ATP, 5 μM of P^{32}-pyrophosphate (30,000 c.p.m./μM) 5 μM of $MgCl_2$ and 100 μM of tris buffer in 0.5 ml. Reaction mixture was incubated at 30° for 10 minutes.

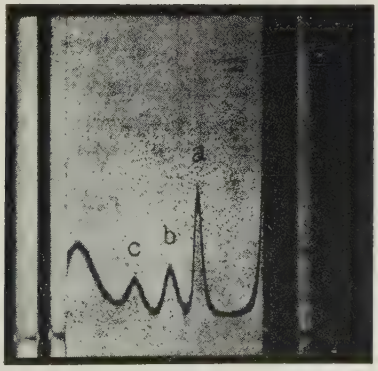


FIG. 5. Sedimentation pattern of WS fraction.

Patterns were recorded 82 minutes after attainment of full speed, 20,410 r.p.m. Sedimentation coecfficients were (a) 56s, (b) 46s, (c) 31s.

acid incorporation, the WP fraction is more transparent against the electron beam. This is due to a release of macromolecular components from the P fraction to the supernatant fraction (WS) in the low concentration of $MgSO_4$ in the medium. More than 60 per cent of protein and 75 per cent of ribonucleic acid were released from precipitate fraction (MgP) into the supernatant (WS) as shown in Table II.

Sedimentation analysis of WS fraction showed that the released substances consisted of components with sedimentation coefficients of 56s, 46s and 31s. The 56s particle is considered as a ribonucleoprotein, because, the content of ribonucleic acid and protein was nearly equal.

It was shown that the amino acid dependent POP-ATP exchange activity was associated with the MgP fraction and this activity was found in the WS fraction, and the WP fraction had no activity.

It can be concluded, therefore, that 10^{-2} to 10^{-3} M of $MgSO_4$ in the resuspending medium was essential to maintain the integrity of the MgP preparation.

TABLE III

Oxygen Dependency of the System

Conditions of incubation[1)]	specific activity (c.p.m./μg. tyrosine equiv.)
MgP fraction in O_2 and phosphate buffer	36
MgP fraction in N_2 and phosphate buffer	0.45
MgP fraction in O_2 and tris buffer	1.8

1) Other conditions and estimation of activity were the same as described in the legend of Table I.

TABLE IV

Effect of Inhibitors on Activity of MgP Fraction

Inhibitor \ Concentration	Per cent inhibitions		
	10^{-3} M	2.5×10^{-4} M	2.5×10^{-5} M
2,4,-Dinitrophenol	92	88	—
Arsenite	91	76	30
Sodium cyanide	94	86	62

Conditions of incorporation were the same as described in the legend of Fig. 2 except for the amount of C^{14}-amino acid mixture (50,000 c.p.m.).

Final concentration of inhibitor is in the table.

Per cent inhibition was calculated from the ratio of the reduced activity to the activity of MgP in the absence of inhibitor.

Specific activity of MgP fraction without inhibitors was 4.5 c.p.m./μg.

Requirement of Aerobic Condition—Under the anaerobic condition the incorporation of amino acids into the protein completely ceased.

Well known inhibitors of respiration or oxidative phosphorylation inhibited the amino acid incorporation of this system, and requirement of inorganic phosphate was shown. (Table III, IV)

Addition of ATP had a little effect on the activity and in reversing the inhibition. (Fig. 6) It appeared that oxidative phosphorylation was an integral part of the MgP system.

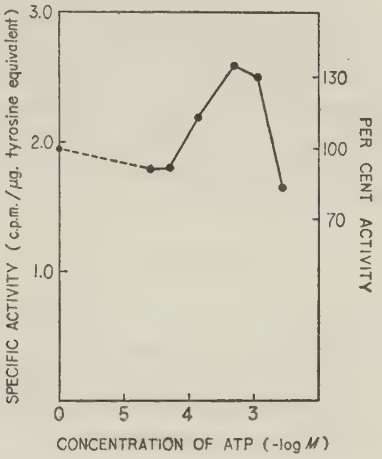


FIG. 6. Effect of ATP concentration on activity.

MgP was incubated with C^{14}-amino acid mixture (10,000 c.p.m.) in the various concentrations of ATP. Other components of incorporation medium were the same as the legend of Fig. 2.

Per cent activity was calculated in the same way as in Table IV.

DISCUSSION

In our investigation it was found that there was a correlation between the activity of amino acid incorporation and the concentration of magnesium ion in the resuspending medium. That is, in the low concentration of magnesium ion, activity of the amino acid incorporation was lost. It was observed that the ribonucleoprotein particles were released from the precipitate fraction (MgP), as well as the enzymes and ribonucleic acids in the presence of low concentrations of magnesium ion.

It has been reported that the ribonucleoprotein particles of bacteria (*11*, *12*, *13*), and yeast (*14*), were stable in the medium of a certain concentration of magnesium ion, and that these particles dissociated to smaller units reversibly when the concentration of magnesium ion was lowered (*11*, *12*, *13*, *15*). In these reports, however, they did not refer to the activities of

ribonucleoprotein particles, though the important role of ribonucleoprotein particles in protein synthesis was known in plants and animals (*16*, *17*).

The loss of activity in the cell-free system of *Pseudomonas P* caused by decreasing the concentration of magnesium ion was not reversible.

It was considered that the inactivation of the system was caused from denaturation of ribonucleoprotein particles in the low concentration of magnesium ion.

This preparation is too complex to study the role of each component (*i.e.* ribonucleoprotein particles, amino acid activating enzymes and oxidative phosphorylation systems) in protein synthesis. Therefore, further fractionation of the preparation is now in progress.

Preliminaly experiments showed that ribonucleoprotein particles obtained from the WS fraction by centrifugation at $105{,}000 \times g$ had no activity by itself, and that the activity was partially recovered when the system was reconstructed with the WP fraction and the concentrated WS fraction which contained ribonucleoprotein particles.

SUMMARY

1. Preparation of subcellular fraction from *Pseudomonas P* and their activities of amino acid incorporation were investigated.

2. Concentration of $MgSO_4$ in the resuspending medium was closely related to the activity of the preparation. Optimal concentration of magnesium ion was $10^{-2}M$, and the preparation lost its activity at the concentration of less than $10^{-4}M$.

3. A large amount of RNA and protein were released from the precipitate fraction (MgP) in the absence of magnesium ion.

4. Amino acid incorporation occurred only under the aerobic condition. The behaviour against various inhibitors suggested that the amino acid incorporation was closely related to the oxidative phosphorylation in this system.

5. The effect of magnesium ion on both the ribonucleoprotein particles and this system was discussed.

The authors wish to express their thanks to Mr. S. Yamamoto for ultracentrifuge analysis and to Mr. D. Okuno for electron micrography.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

REGULATORY EFFECT OF MALONATE ON GLUCOSE METABOLISM IN HUMAN EARLIER PLACENTA*

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(Received for publication, March 5, 1960)

In past years, malonate has extensively been used in mammalian systems *in vitro* as an inhibitor of the tricarboxylic acid cycle with very little attention to its metabolism *per se*. Using human earlier placenta, we found that malonate was a poor inhibitor of endogenous respiration (*2*). A further investigation indicated that malonate decreased the oxygen consumption of human earlier placenta below the level of endogenous respiration when glucose was added as a substrate (*3*).

Recently, Nakada, Wolfe and Wick (*4*) and Hosoya and Kawada (*5*) have independently reported on the enzymatic decarboxylation of malonate in mammalian systems by rat tissues and by human earlier placenta. On the other hands, Brady (*6*) and Wakil (*7*) demonstrated that malonate derivatives were intermediates of fatty acid synthesis from acetate.

The present studies indicate that malonate can exert a regulatory effect on the glucose metabolism of human earlier placenta and that malonate itself is an active metabolic substrate in mammalian systems.

MATERIALS AND METHODS

Human earlier placentas (gestational age 13–20 weeks) were obtained at therapeutic interruptions of pregnancy performed for various medical and surgical indications. The tissue was placed in ice-cold Ringer solution and transfered to the laboratory. The tissues were sliced or homogenized as soon as the tissue could be obtained.

In the tissue slice experiments, free hand slices of earlier placenta weighing about 300 mg. were incubated in a media containing 1.5 ml. of Krebs-Ringer phosphate buffer (pH 7.4). The incubations (total volume 3.0 ml.) were carried out in conventional Warburg vessels with 100 per cent oxygen as the gas phase and alkali present in the center wells. 40 μmoles of glucose and 60 μmoles of potassium malonate were placed in the side arm of the flask and tipped into the main reaction chamber after the vessels were gassed with oxygen.

* This report was presented at the 32nd Meeting of the Japanese Biochemical Society, on November 3, 1959 in Osaka (*1*).

This work was supported in part by a grant for scientific research from the Ministry of Education.

After a two hour incubation at 37° the slices were removed, weighed, and aliquot of the incubation medium was analyzed for lactate (*8*). In those experiments in which uniformly labeled glucose and potassium malonate-2-C^{14} (each counts *ca.* 5,000 c.p.m. per μmole) were used, the respiratory carbon dioxide was recovered from the center well alkali (0.2 ml. of 5 per cent carbon dioxide-free solution of sodium hydroxide), converted to barium carbonate, and counted with a windowless, proportional flow counter (*9*). The extraction of total lipids from slices for C^{14} analysis was carried out by the method of Folch (*10*) and the isolation of glycogen from slices by the method of Villee (*9*).

In the homogenate experiments each vessel contained 3.0 ml. of an incubation mixture composed of 0.01 μmoles of cytochrome c, 0.75 μmoles of diphosphopyridine nucleotide, 2.5 μmoles of adenosine triphosphate, 10 μmoles of potassium chloride, 10 μmoles of magnesium chloride, 50 μmoles of phosphate buffer (pH 6.9), 40 μmoles of glucose, 60 μmoles of potassium malonate, plus 1.0 ml. of 20 per cent human earlier placental homogenate in 0.25 *M* sucrose. The buffer was adjusted to pH 6.9 and did not differ from this value more than ± 0.1 at the end of the two hour incubation period. The gas phase was 100 per cent oxygen and alkali was present in the center wells.

The homogenates were prepared in a smooth glass homogenizer fitted with a Teflon pestle and contained 20 per cent (weight per volume) human earlier placenta in 0.25 *M* sucrose. After homogenization for not more than 30 seconds at ice bath temperature, these preparations were centrifuged for 2 minutes at $100 \times g$ in a small, angle head centrifuge in the cold room and the free flowing fluid was delivered into the vessels. Each vessel contained about 2.0 mg. of nitrogen.

RESULTS

Oxygen Consumption of Tissue Slice—Oxygen consumptions of human earlier placenta in the presence of various substrates and malonate are shown in Table I. Endogenous respiration was not affected with addition of either

TABLE I

Oxygen Consumption of Human Earlier Placental Slice

	No. of experiments	Malonate	
		absent	present
No addition	40	3.02±0.24	2.94±0.31
Glucose	40	3.22±0.40	2.25±0.19
Ribose	6	3.10±0.30	2.00±0.18
Fructose-1,6-diphosphate	6	3.03±0.33	2.14±0.22
Sodium pyruvate	5	3.34±0.42	2.65±0.23
Sodium acetate	5	3.04±0.35	3.02±0.25

The values are in microliters of oxygen consumed per mg. of dry weight of tissue per hour ± the standard error of the mean. Malonate, 60 μmoles; others, 40 μmoles.

Human earlier placenta, gestational age 10 to 16 weeks.

glucose or malonate. However, addition of a mixture of glucose and malonate as a substrate decreases oxygen consumption to the degree of about three

fourth of the endogenous respiration. This result indicates that malonate exerts an inhibitory effect on the oxygen consumption in the presence of glucose. Further experiment was carried out using ribose, fructose-1,6-diphosphate, pyruvate and acetate instead of glucose. With the addition of malonate to ribose or to fructose-1,6-diphosphate, oxygen consumption is decreased to about two third or three fourth of endogenous respiration. On the other hand, in the case of addition of malonate to acetate, oxygen consumption is not decreased.

Oxygen Consumption of Homogenate—Using human earlier placental homogenate, oxygen consumption in the presence of glucose and malonate was investigated (Table II). Glucose slightly depressed oxygen uptake, whereas malonate increased it. A further more increase of oxygen uptake was

TABLE II

Oxygen Consumption of Human Earlier Placental Homogenate

No substrate	Glucose	Malonate	Glucose, Malonate
11.37±0.82	9.72±0.62	14.4±0.98	15.97±1.35

The values are in microliters per mg. of nitrogen per hour ± the standard error of the mean (8 determinations). Glucose, 40 μmoles; malonate, 60 μmoles.

Human earlier placenta, gestational age 10 to 16 weeks.

TABLE III

Metabolism of C^{14}-Labeled Substrates in Human Earlier Placental Slice

Substrate		No substrate	Glucose-U-C^{14}		Malonate-2-C^{14}	
				Malonate		Glucose
Oxygen consumption		10.27 ±0.64	10.45 ±0.44	7.61 ±0.88	9.50 ±0.47	7.74 ±0.58
lactate production		3.97 ±0.57	10.24 ±0.80	11.62 ±1.33	8.41 ±0.50	11.03 ±0.82
Conversion of isotopic carbon of substrate	to CO_2		0.106 ±0.013	0.038 ±0.006	0.004 ±0.001	0.005 ±0.001
	to glycogen		0.185 ±0.035	0.025 ±0.003	0.060 ±0.008	0.059 ±0.006
	to lipid		0.090 ±0.005	0.019 ±0.002	0.018 ±0.003	0.014 ±0.002

All values are expressed as μmoles per g. of wet weight of tissue per hour ± the standard error of the mean (6 determinations). Glucose, 40 μmoles; malonate, 60 μmoles. Human earlier placenta, gestational age 15 to 20 weeks.

observed with a mixture of glucose and malonate. This result indicates that malonate metabolizing enzyme system is present in human earlier placenta.

However, in the Thunberg's tube experiments succinic dehydrogenase activity of this homogenate is inhibited competitively with malonate (*5*).

Metabolism of Glucose and Malonate—A further investigation was carried out to elucidate the metabolism of glucose and malonate in tissue slices of human earlier placenta using uniformly labeled glucose and malonate-2-C^{14} (Table III). Lactate productions in the presence of malonate or glucose are two or two and half times of the no addition. In a mixture of glucose and malonate, lactate production increased further.

When glucose-U-C^{14} is used as a substrate, the rates of carbon dioxide production, glycogenesis and lipid synthesis are decreased with the addition of malonate to about a third, a seventh and a quarter of the control respectively. On the other hand, glyconeogenesis and lipid synthesis from malonate-2-C^{14} are observed, and the addition of glucose has no effects on these metabolic rates.

DISCUSSION

Malonate has been considered to be a competitive inhibitor of the succinic dehydrogenase. In this two or three years, however, its metabolism in mammalian systems has been elucidated by several workers (*4–7*). Human placenta also has an enzyme system concerning malonate metabolism (*5*).

Above experiments show that the addition of sugar and malonate inhibited oxygen consumption of tissue slice and increased oxygen uptake of homogenate. These phenomena might be due to the cell membrane permeability, or to the addition of cofactors in homogenate experiments. This problem is being investigated.

Isotopic experiments with slice present the results that malonate inhibits the glucose metabolism and malonate itself is metabolized in human earlier placenta.

From the fact that malonate as shown in the Thunberg's tube experiments inhibits competitively succinic dehydrogenase activity, malonate might have an inhibitory action to the tricarboxylic acid cycle. The decrease of carbon dioxide production from glucose with malonate might be due to this reaction. On the other hand, in the inhibitory effects of malonate on glycogenesis and lipid synthesis from glucose, more complicated metabolic reactions might be involved.

At any rate, malonate would be not an ordinary inhibitor but an active metabolic substrate having a regulatory action in living cells.

SUMMARY

1. Metabolism of human earlier placental slice in the presence of glucose and malonate was observed.

Oxygen consumption is decreased, and aerobic lactate production is increased compared with no addition.

Glycogenesis, lipid synthesis and carbon dioxide production from glucose

U-C^{14} are decreased with malonate.

Those metabolisms from malonate-2-C^{14} are not affected with glucose.

2. Oxygen consumption of human earlier placental homogenate, in glucose plus malonate, is on a higher level than in either glucose or malonate alone.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

METABOLISM OF L-LYSINE BY BACTERIAL ENZYMES

II. L-LYSINE OXIDASE*

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(Recieved for publication, March 10, 1960)

The metabolism of lysine is relatively obscure compared with that of other amino acids, because, as reported by Schoenheimer *et al.* (*2*, *8*), lysine is not reversibly deaminated. Moreover neither L- nor D-amino acid oxidase shows significant activity with this amino acid (*3*, *4*) and there is no known transaminase for lysine (*5*, *6*). Some possible intermediates, such as δ-aminovaleric acid (*7*), glutaric acid (*9*) and pipecolic acid (*2*) were suggested, but there was no direct evidence for any of them.

The first step towards the clarification of the pathway of lysine metabolism was reported by Borsook *et al.* They found an appreciable amount

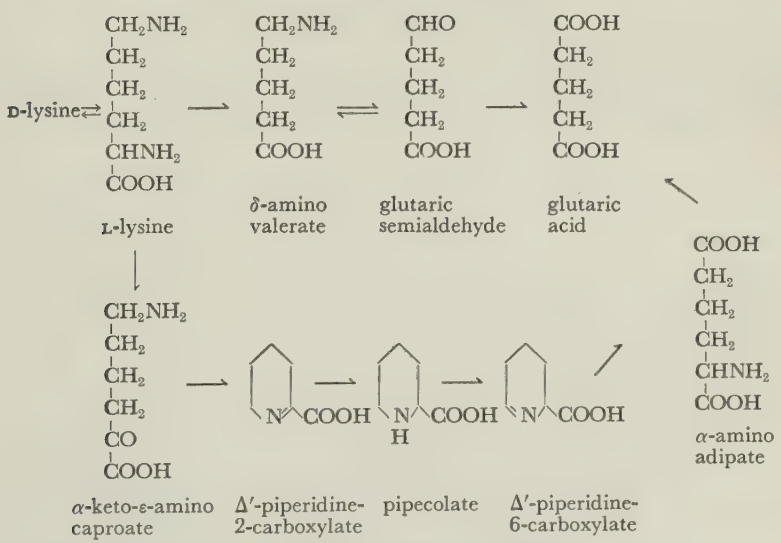

FIG. 1. Metabolic pathway of L-lysine.

of radio-activity in α-aminoadipic acid when lysine-6-C^{14} was incubated with a guinea pig liver homogenate (*10*). They also reported that α-aminoadipic

* The first of this series of papers (*20*) is designated as I.

acid is converted to glutaric acid (*11*). Altman *et al.* (*12*) obtained similar results. Although Borsook *et al.* postulated that the ε-amino group of lysine may be liberated first, in 1954 Rothstein and Miller found, from *in vivo* experiments with rats, that lysine is first deaminated in the α-position (*13*) forming pipecolic acid (*14*). Boulanger and Osteux isolated a basic amino acid oxidase from turkey liver which oxidized lysine to Δ'-piperidine-2-carboxylic acid (*17*). This carboxylic acid has more recently been found to be converted to pipecolic acid (*15*), which can be further metabolized to α-amino adipic acid (*16*). A summary of a scheme of lysine metabolism based on the above results is shown in Fig. 1.

Pipecolic acid has been isolated as an intermediate in the catabolism of lysine in *Neurospora* (*19*) and green beans (*21*) as well as in animals.

In 1954, we also reported on the metabolism of lysine by an enzyme isolated from lysine adapted *Pseudomonas.* The action of this enzyme was unlike that given in the previous reports mentioned above, for it was found that lysine was oxidized with an accompanying equimolecular oxygen uptake, and ammonia and carbon dioxide liberation, to δ-amino-valeric acid, but that pipecolic acid was not formed (20).

The present paper reports on the purification of this L-lysine oxidase from *Pseudomonas* and shows that the nature of this oxidase differs from that of other known amino acid oxidases.

METHODS AND MATERIALS

Acetone Powder of L-*Lysine Oxidase Adapted Pseudomonas*—The strain of *Pseudomas* used for this work was the same as that used previously (*20*). The method of adaptation of the cells and preparation of the acetone powder was similar to that reported previously (*20*).

Assay of Enzyme Activity—The reaction mixture contained 60 μmoles of phosphate buffer (pH 8.0), 1 μmole of glutathione, 10 μmoles of L-lysine and 0.2 ml. of the enzyme preparation to be assayed. The total volume was adjusted to 2 ml. with distilled water. The activity of the enzyme is expressed in this paper as μliters of oxygen consumption per hour at 30°. Oxygen consumption and carbon dioxide liberation were measured by the conventional Warburg manometric technique. Ammonia was measured in a Conway apparatus (*22*) and protein by Folin's reagent (*23*). When L-lysine-U-C^{14} was used as substrate, the reaction was stopped by addition of 0.2 ml. of 50 per cent perchloric acid from the side arm of the Warburg vessel. Carbon dioxide was trapped in 0.2 ml. of 20 per cent potassium hydroxide in the center well of the vessel. It was then converted to barium carbonate and counted, radioactivity being measured in a Geiger-Müller counter (*24*). The reaction mixture was deproteinized and neutralized and the supernatant was acidified with dilute hydrochloric acid and evaporated to dryness. The residue was suspended in 1 ml. of ethyl alcohol and after removal of insoluble material, 2 μmoles of δ-aminovaleric acid was added. 0.1 ml. of this solution was applied to a paper chromatogram and spots were developed with the same solvent as previously (*20*). The paper chromatogram was sprayed with ninhydrin and the location and intensity of the radioactivity measured.

Crystalline catalase from hog liver was generously given by Prof. K. Okunuki of

the Department of Biology, and flavin adenine mono- and dinucleotides by Prof. H. Kubo, of the Department of Physiology of this University. α-Ketolysine was synthesized by Dr. F. Suzuki, of this Department.

RESULTS

Purification of L-*Lysine Oxidase*—Ten g. of the acetone powder prepared from *Pseudomonas* adapted to L-lysine, were homogenized with 400 ml. of distilled water containing 0.1 per cent β-mercaptoethanol. The homogenate was stored overnight in a refrigerator. After centrifugation, the supernatant (Extract) was mixed with one tenth of its volume of 5 per cent streptomycin solution, and the mixture stirred for ten minutes and then centrifuged. The supernatant (Streptomycin Treatment) was brought to 40 per cent saturation with solid ammonium sulfate and the mixture stirred for 30 minutes after adjusting the pH to 7.4 with 10 per cent ammonia. After centrifugation, more ammonium sulfate was added to 60 per cent saturation. The mixture was centrifuged and the precipitate dissolved in 50 ml. of distilled water. It was dialyzed overnight against 2 liters of distilled water containing 0.1 per cent β-mercaptoethanol. Calcium phosphate gel was added to the dialyzate (Ammonium sulfate fractionation I) at a rate of 1 mg. dry weight gel per mg. protein. After adjusting the pH to 5.7, the mixture was stirred for 20 minutes and then centrifuged. The enzyme was eluted from the gel by stirring with 20 ml. of 0.2 *M* phosphate buffer (pH 8.0). The eluate (Ca-gel treatment) was fractionated again with ammonium sulfate. The fraction precipitating between 45 and 55 per cent saturation was dialyzed in the same way as previously. About 10 ml. of dialyzate were obtained and used as the enzyme preparation (Ammonium sulfate fractionation II). This preparation showed an increase of 20 fold in specific activity over the Extract and there was a 50 per cent yield, as shown in Table I.

TABLE I

Purification of L-*Lysine Oxidase*

	Total Protein	Specific Activity	Total Activity
	mg.	μliter/mg./hour	mliter/hour
Extract	1200	66.5	77
Streptomycin treatment	1100	71.2	78
Ammonium sulfate fractionation I	305.4	185.5	56.5
Ca-gel treatment	76.9	533.0	41
Ammontum sulfate fractionation II	29.6	1308.7	38.5

Three ml. of this preparation were subjected to starch column electrophoresis. Some fractions showed about a 100 fold increase in specific activity, but the yield was only 10 to 15 per cent, as shown in Fig. 2. Therefore

in most experiments, enzyme was used without treatment by electrophoresis. All the purification was carried out at below 4°.

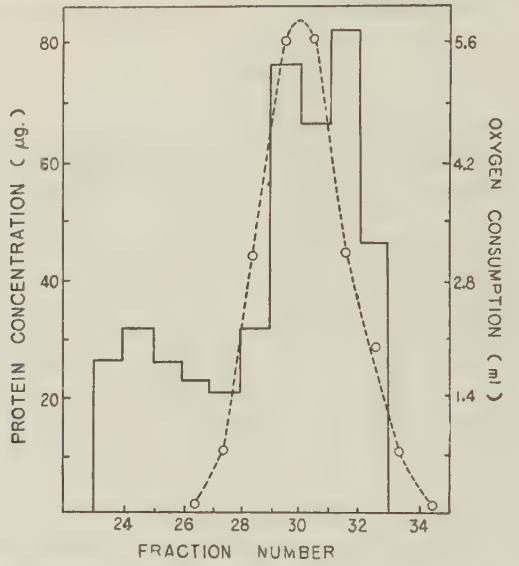

Fig. 2. Starch column electrophoresis of L-lysine oxidase. Three ml. of the protein solution was chromatographed on a starch column (5×30 cm.) at 600 volts and 20–22 mA. for 19 hours and eluted with $1\times10^{-2}M$ phosphate buffer (pH 7.4). The fraction size was 3 ml. The solid line represents protein concentration and the dotted line the L-lysine oxidase activity.

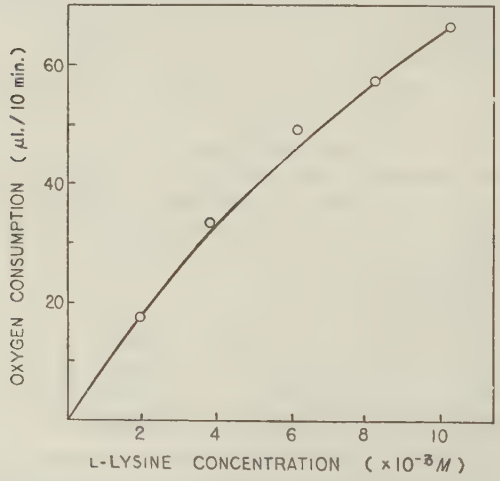

Fig. 3. Oxidase activity at different concentrations of L-lysine.

Substrate Specificity—Ten μmoles of the following DL-amino acids were tested with the enzyme: alanine, serine, cysteine, glycine, valine, leucine,

isoleucine, methionine, tryptophan, phenylalanine, histidine, α-γ-diamino butyric acid, ornithine, arginine, glutamic acid, aspartic acid, and D- and L-lysine. There was only oxygen uptake in the presence of L-lysine.

The oxygen consumption in the presence of different concentrations of L-lysine is shown in Fig. 3. From this the K_m was calculated as $9.1 \times 10^{-4} M$.

Inhibitors—Of the inhibitors tested only *p*-chloromercuribenzoate had an effect. It caused 100 per cent inhibition at a concentration of $1 \times 10^{-4} M$. The following inhibitors were without effect at a concentration of $1 \times 10^{-3} M$: semicarbazide, hydroxylamine, KCN, arsenate, cupferon, *o*-phenanthroline, 8-hydroxyquinoline and nitroso-R.

The inhibitory effect of *p*-mercuribenzoate suggests that a sulfhydryl group on the enzyme protein is important for enzyme activity. This is supported by the observation that, when the reaction mixture contains no glutathione, there is considerably less activity, as shown in Fig. 4.

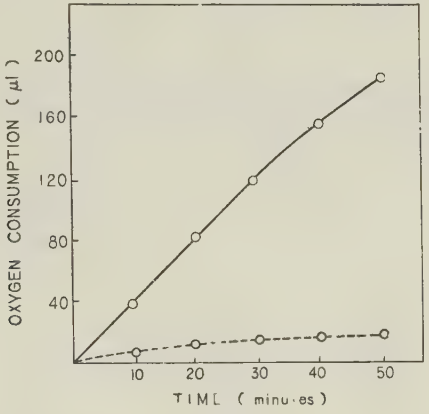

FIG. 4. Effect of glutathione on oxidase activity. The solid line represents oxidase activity on addition of glutathione and the dotted line that with no glutathione.

Effect of Flavins and Dyes as Electron Acceptors—There was no observable increase in activity when either flavin adenine mono- or dinucleotide was added to the reaction mixture at a concentration of $1 \times 10^{-5} M$. The enzyme preparation after electrophoresis showed no evidence of an absorption spectrum of flavin.

This preparation did not reduce any of the dyes tested (*i. e.* methylene blue, 2,6,-dichlorophenol indophenol, neutral red or TTC). Addition of crystalline catalase at a concentration of 0.1 mg. per 2 ml. did not reduce the oxygen consumption.

Stoichiometry—Previously the authors reported that L-lysine is converted to δ-aminovaleric acid with an accompanying equimolecular oxygen consumption and ammonia and carbon dioxide liberation (*20*). However from the previous work it was uncertain whether α-keto-ε-aminocaproic acid was an

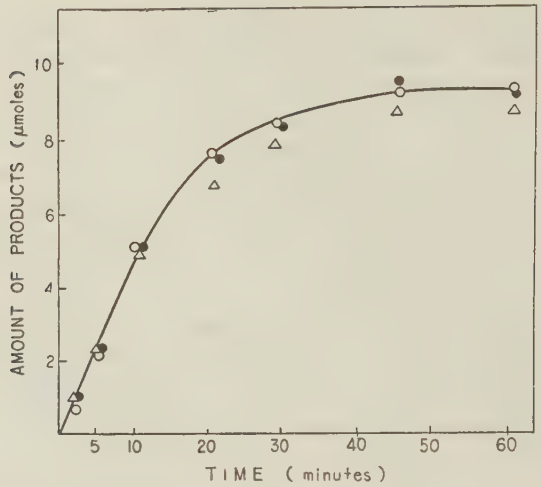

FIG. 5. Stoichiometry of oxidase reaction. A) oxygen consumption and ammonia and carbon dioxide liberation.
○ represents the oxygen consumption, △ the ammonia, and ● the carbon dioxide liberation.

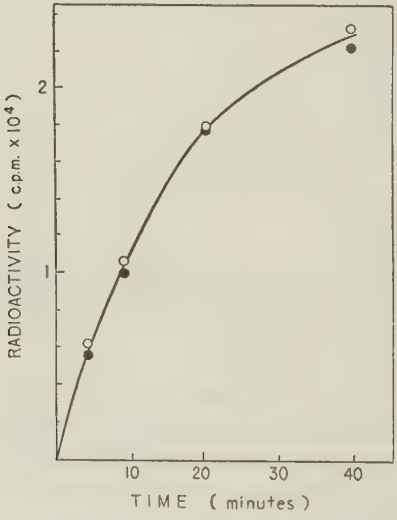

FIG. 6. Stoichiometry of oxidase reaction. B) Formation of δ-aminovaleric acid and carbon dioxide from L-lysine-U-C^{14}.

The reaction system was as described in the text, except 1 mg. of L-lysine-U-C^{14} (5 μc.) and 0.05 ml. of enzyme solution were added. The solid circle represents the radioactivity of the carbon dioxide and open circle that of δ-aminovaleric acid as calculated from the paper chromatogram shown in Fig. 7.

intermediate. A synthetic sample of the keto acid was tested as substrate for the enzyme. No oxygen consumption was observed.

Fig. 5 shows the time course of the oxygen uptake and the liberation of ammonia and carbon dioxide from L-lysine. The concomitant formation of δ-aminovaleric acid from L-lysine-U-C^{14} is shown in Fig. 6. In these experiments formation of carbon dioxide, ammonia, and δ-aminovaleric acid and oxygen consumption from L-lysine were equimolecular throughout the reaction period. Moreover no significant radioactivity was found by paper chromatography other than that in the δ-aminovaleric acid and the residual lysine, as shown in Fig. 7.

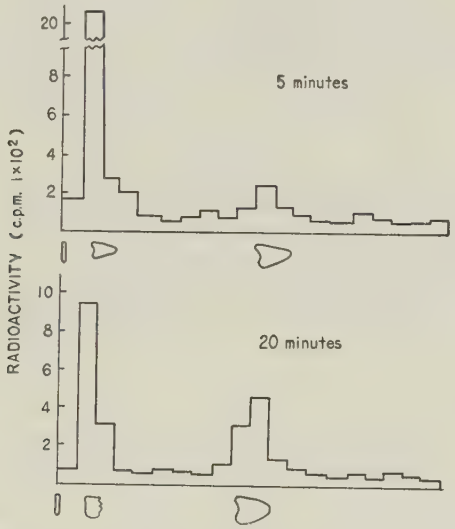

FIG. 7. Proof of the formation of δ-aminovaleric acid from L-lysine-U-C^{14} by paper chromatography.

The reaction system was the same as for Fig. 6. The ninhydrin positive spots shown above are from left to right: origin, lysine and δ-aminovaleric acid.

Identification of the End Product of the Reaction—A mixture of 200 mg. of L-lysine, 4 mmoles of Tris buffer (pH 8.0), 10 μmoles of glutathione and 30 ml. of enzyme solution (Ammonium sulfate fractionation I) in a total volume of 100 ml. was incubated with shaking at 30°. The reaction was followed by measuring the concentration of ammonia in the reaction mixture. After the reaction had stopped at the end of 3 hours, 6 ml. of 50 per cent perchloric acid was added to the mixture. The supernatant was neutralized with 5 *N* potassium hydroxide. The insoluble material was removed by centrifugation and the supernatant was concentrated to 5 ml. and applied to an Amberlite IRC-50 (Na^+ form) column (2×10 cm.). The ninhydrin positive fractions were collected and dried. The residue was acidified with a few drops of dilute hydrochloric acid and the 30 ml. of ethanol was added and the inso-

luble material removed by filtration. Fifty ml. of ether was slowly added and the fine crystals thus formed were collected. After recrystallization in the same manner, 45.5 mg. of crystalline material was obtained. Chemical analysis of the crystals coincided with that of authentic δ-aminovaleric acid.

$C_5H_{11}O_2NHCl$ (m. w. 152.5)

Calculated	C	39.09	H	7.82	N	9.12	m. p. 91 (*25*)
Found		39.01		8.12		9.20	92–93°

DISCUSSION

Although there has been much work on the subject, the metabolism of lysine has remained relatively unknown. In 1913, Dakin reported that lysine was neither glycogenic nor ketogenic in phlorizinized dogs (*1*) and this finding was confirmed by other workers (*27*, *28*). Weissman and Schoenheimer (*2*) and Clark and Rittenberg (*8*) using isotopic lysine, found that it was not reversibly deaminated *in vivo*. In fact, neither L-nor D-amino acid oxidase oxidizes lysine (*3*, *4*, *26*), and further more there is no evidence for active transamination of lysine (*5*, *6*). However, lysine acetylated or carboxybenzoated at the ε-amino group is a substrate for amino acid oxidase (*7*, *29*). Recently Boulanger and Osteux isolated an L-amino acid oxidase from turkey liver, which preferentially oxidizes the basic amino acids such as arginine, ornithine, histidine and lysine (*17*, *18*).

From the facts mentioned above, the L-lysine oxidase reported here is of particular interest. It is specific for L-lysine and is not affected by flavine and catalase. Therefore it probably is not a flavoenzyme and free hydrogen peroxide does not participate in the reaction. Thus the following reaction, in which F represents a flavoprotein, does not occur:

$$\text{Lysine} + F + H_2O \longrightarrow \alpha\text{-keto-}\varepsilon\text{-amino caproate} + FH_2 + NH_3$$
$$FH_2 + O_2 \longrightarrow F + H_2O_2$$
$$\alpha\text{-keto-}\varepsilon\text{-amino caproate} + H_2O_2 \longrightarrow \delta\text{-amino valerate} + CO_2 + H_2O$$

Another possible mechanism is the complex reaction of two enzymes, lysine oxidase and ketolysine oxidative decarboxylase:

$$\text{Lysine} + {}^1/_2O_2 \longrightarrow \alpha\text{-keto-}\varepsilon\text{-amino caproate} + NH_3$$
$$\alpha\text{-keto-}\varepsilon\text{-amino caproate} + {}^1/_2O_2 \longrightarrow \delta\text{-amino valerate} + CO_2$$

However 1) synthetic α-keto-ε-aminocaproic acid was not a substrate for the enzyme, 2) there was equimolecular formation of the reaction products and oxygen consumption during the reaction, and 3) no carbonyl reagents tested inhibited the reaction. All three results are incompatible with the above reaction mechanism.

Sutton reported on lactic oxidative decarboxylase of *Mycobacterium phlei* which has many similarities in its mode af action to L-lysine oxidase (*37*, *38*).

$$CH_3CHOHCOOH + O_2 \longrightarrow CH_3COOH + CO_2 + H_2O$$

In this reaction one mole of oxygen is consumed and one mole of acetate and carbon dioxide formed. Pyruvic acid is not an intermediate, and added catalase does not inhibit the reaction. Lactic oxidative decarboxylase and

L-lysine oxidase however differ in that the former is a flavoprotein.

A third possible reaction is catalyzed by a single enzyme and may involve molecular oxygen. Experiments using O_2^{18} and H_2O^{18} are now being planned, and the enzyme is being purified further.

Mammals and plants metabolize lysine to glutaric acid (*11*, *12*, *30*) through pipecolic acid (*14*, *19*). However in *Pseudomonas* as reported here, lysine is oxidized to δ-aminovaleric acid which is further metabolized to glutaric acid (*31*), as shown in Fig. 1. Though δ-aminovaleric acid was reported to be metabolized to glutaric acid in rats (*32*), little evidence was found for its formation *in vivo* in animals (*30*). In *Clostridium* δ-aminovaleric acid has been shown to be formed from proline (*33*, *34*).

The *Pseudomonas* used for these experiments also contains a lysine racemase (*35*) and it is possible to use this bacteria for the differential determination of D- and L-lysine (*36*).

SUMMARY

1. L-Lysine oxidase was isolated and purified from *Pseudomonas* bacteria.
2. This enzyme specifically oxidizes L-lysine. The reaction is as follows:

L-Lysine + O_2 ⟶ δ-aminovaleric acid + CO_2 + NH_3

3. No effect of catalase, flavin or dyes was observed. This new type of L-lysine oxidase is discussed.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

METABOLISM OF L-LYSINE BY BACTERIAL ENZYMES

III. LYSINE RACEMASE

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(Received for publication, March 16, 1960)

During the study of L-lysine oxidase in *Pseudomonas*, as reported previously (*1*), it was observed that a crude extract of an acetone powder of the bacteria could oxidize both D- and L-lysine. However the oxidation of D-lysine was completely inhibited by hydroxylamine. This observation suggested the presence of a lysine recemase in the extract. Huang *et al.* (*2*) reported briefly on the occurrence of such a racemase in *Proteus vulgaris* but the details of the properties of their preparation were not given.

This paper reports the purification of lysine racemase from *Pseudomonas* and its mode of action.

MATERIALS AND METHODS

Preparation of the Acetone Powder of Pseudomonas—The bacterial strain of *Pseudomonas* used for this work was the same as that used previously (*1*). The acetone powder was prepared in the same way as previously described but the bacteria used had not adapted to lysine because it was found that adaptation did not affect the activity of the racemase.

Assay Method—10 μmoles of D-lysine, 80 μmoles of phosphate buffer (pH 8.0) and 0.2 ml. of the enzyme solution in a total volume of 2 ml. was incubated anaerobically in a Thunberg tube for 20 minutes at 30°. The reaction was stopped by heating the tube in a boiling water bath for 5 minutes. Then 1 ml. of L-lysine oxidase solution was incubated with 1 ml. of this reaction mixture in a conventional Warburg manometer at 30°, and the amount of L-lysine which had been formed was calculated from the oxygen comsumption. The activity of the racemase was expressed as the μmole of L-lysine formed in 20 minutes. The L-lysine oxidase solution contained per ml.: 1 μmole of glutathione, 30 μmoles of hydroxylamine, 60 μmoles of phosphate buffer (pH 8.0) and 15 mg. of acetone powder of cells adapted to L-lysine as reported previously (*1*).

When L-lysine was used in place of D-lysine, the activity of the racemase was calculated from the amount of L-lysine which remained after incubation.

Protein was measured by Folin's method (*13*).

L-Lysine was kindly given by Sumitomo Chemical Co. and D-lysine was prepared from acetyl-benzoyl-D-lysine which was generously supplied by Tanabe Pharmaceutical Co. Pyridoxal phosphate was obtained from the California Foundation.

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RESULTS

Purification of Lysine Racemase—Ten g. of acetone powder were homogenized with 400 ml. of 2×10^{-2} *M* phosphate buffer (pH 7.4) and the mixture allowed to stand overnight in a cold room. The homogenate was then centrifuged at $15{,}000\times g$ for 15 minutes. One tenth volume of 5 per cent streptomycin was added to the supernatant (Extract) and the resulting precipitate removed by centrifugation. Solid ammonium sulphate was added to the supernatant (Streptomycin treatment) to 40 per cent saturation. After centrifugation, further ammonium sulfate was added to the supernatant to 60 per cent saturation. During the addition of the ammonium sulfate, the pH was maintained at 7.4 by addition of 10 per cent ammonia solution. The fraction precipitating between 40 to 60 per cent saturation was dissolved in 40 ml. of 7×10^{-3} *M* phosphate buffer (pH 7.4) and dialyzed against 3 liters of the same buffer. The dialyzed solution (Ammonium sulfate fraction I) was heated to 50° and then after 5 minutes was quickly cooled to 0–4° and centrifuged. To the supernatant (Heat treatment) Ca-phosphate gel was added in a ratio of 1 mg. protein to 0.5 mg. gel (dry weight) and the pH was adjusted to 5.7 with 0.1 *N* acetic acid. After 15 minutes the solution was centrifuged and the supernatant (Ca-gel treatment) was again brought to 50 per cent saturation with ammonium sulfate. The resulting precipitate was dissolved in 20 ml. of buffer and dialyzed as previously. This dialyzate was used as the enzyme solution (Ammonium sulfate fraction II).

The activity of the enzyme at each step in the purification is shown in

TABLE I

Purification of Lysine Racemase

	Specific activity	Total activity	Total activity of L-lysine oxidase
	μmole/mg./min.	μmole/min.	μliter/min.
Extract	0.066	136.5	360
Streptomycin treatment	0.146	170.0	360
Ammonium sulfate fraction I	0.360	105.9	330
Heat treatment	0.271	83.7	20
Ca-gel treatment	0.484	48.3	7.7
Ammonium sulfate fraction II	0.805	23.1	1.1

The activity of L-lysine oxidase was measured as reported previously (*11*). The specific activity of the racemase was expressed as the μmoles of L-lysine formed per minute per mg. enzyme protein.

Table I. About a 10 fold increase in specific activity was achieved and the final preparation contained insignificant L-lysine oxidase activity.

Preliminary Experiments—The enzyme preparation at the stage of Extract showed oxidase activity with both L- and D-lysine, as seen in Fig. 1. The

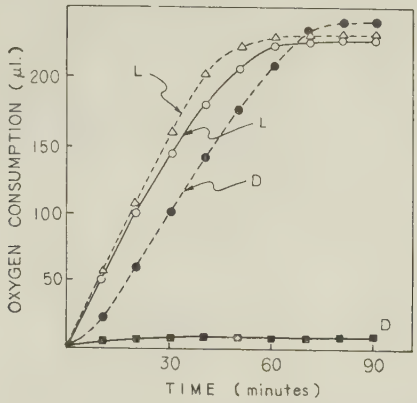

FIG. 1. Oxygen consumption by the crude extract with D- and L-lysine and the effect of addition of hydroxylamine.

The reaction system contained 10 μmoles of D- or L-lysine, 60 μmoles of phosphate buffer (pH 8.0) and 0.5 ml. of the extract with or without 30 μmoles of hydroxylamine. The volume was adjusted to 2 ml. with distilled water. The oxygen uptake was measured in a Warburg manometer. L shows the reaction with L-lysine and D with D-lysine as substrate. The solid line shows the reaction with, and dotted line without hydroxylamine.

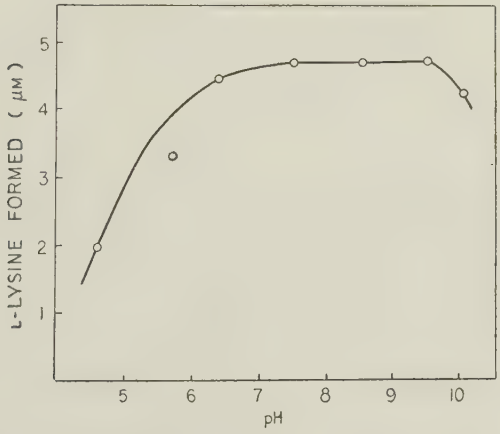

FIG. 2. Optimal pH.

Below pH 6, acetate buffer, pH 6 to 8, phosphate buffer, and above pH 8, borate buffer. 60 μmoles of each buffer was used.

oxygen consumption was the same with either isomer, although there was a slight lag phase with D-lysine. Hydroxylamine completely inhibited oxygen

consumption with the D-, but had no effect with the L-isomer. The occurrence of a lag phase and the inhibition by hydroxylamine of D-lysine oxidation strongly indicated the presence of a lysine racemase converting the D- to the L-form, before the later was oxidized.

Optimal pH and Substrate Concentration—The enzyme had a fairly broad pH optimum, as shown in Fig. 2. On the acid side activity decreases sharply, while there was a gradual decline on the alkaline.

As shown in Fig. 3 the optimal concentration of both D- and L-lysine was about 30 μmoles.

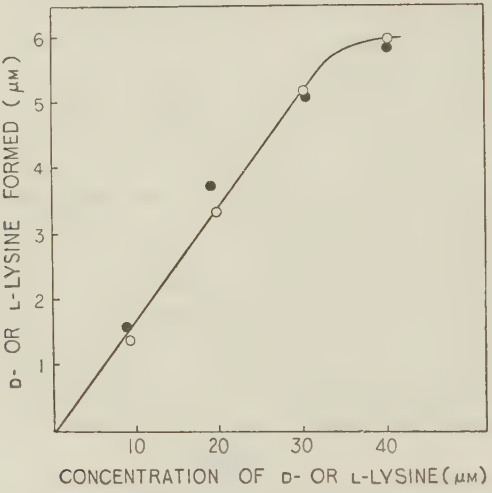

FIG. 3. Optimal concentration of D- or L-lysine as substrates. The open circles show the L-lysine formed from D-lysine, and solid circles the D-lysine from L-lysine.

Reversibility of the Reaction—D- And L-lysine were incubated for different periods with the enzyme and the L-lysine formed or remaining, respectively, were found to be as shown in Fig. 4. The reaction ceased when 50 per cent of either isomer had been converted to the other.

Direct Evidence for Enzymatic Racemization of Lysine—Two hundred mg. of L- and D-lysine were separately incubated with the enzyme and the reaction stopped at the time shown in Fig. 5 by addition of 50 per cent perchloric acid to a final concentration of 3 per cent. After centrifugation, the supernatant was neutralized with 5 *N* KOH and centrifuged. The supernatant was concentrated to 3 ml. and then introduced into an Amberlite IRC 50 (Na^+ form) column (1×10 cm.). After washing the column with water, lysine was eluted with 1 *N* HCl. Ninhydrin positive fractions were pooled and made up to 20 ml. with 6 *N* HCl. The optical rotation was measured and the amino acid concentration measured by the method of Moore and Stein (*3*). Thus the $[\alpha]_D^{20}$ was calculated. The racemization of the optical

rotation coincided with the curve shown in Fig. 4 measured by L-lysine oxidase. This proves the validity of the enzymatic assay method.

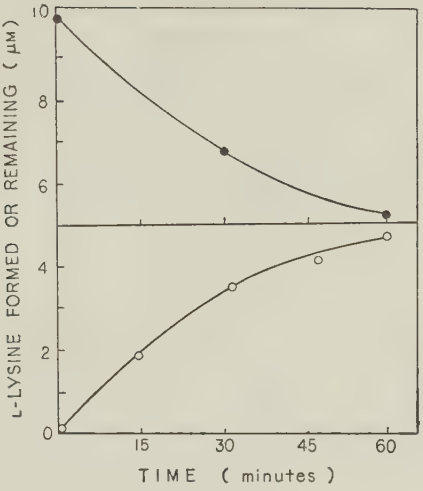

FIG. 4. Reversibility of the reaction.
The open circles represent the formation of L-lysine with D-lysine as substrate and solid circles L-lysine remaining with L-lysine as substrate.

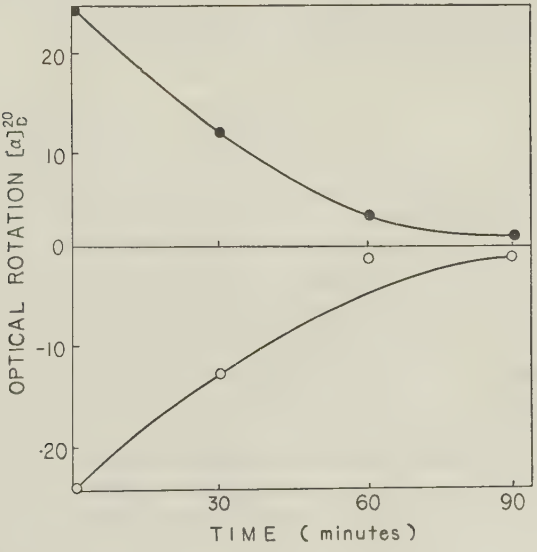

FIG. 5. Racemization of lysine isomers measured by optical rotation. Signs as in Fig. 4.

Inhibitors and Cofactors—Pyridoxal phosphate has been reported to be a cofactor for alanine and glutamic acid recemase (*4*, *5*). However the activity

of the preparation described in this report was not affected by pyridoxal phosphate. Hydroxylamine, at a concentration of 1×10^{-3} *M* completely inhibited activity, but the activity could be recovered by the addition of a

TABLE II

Effect of Various Inhibitors and Pyridoxal Phosphate on Racemase Activity

Inhibitor	Concentration of inhibitor	Pyridoxal phosphaet	Activity
	M	*M*	μmole
	—	—	3.22
Hydroxylamine	1×10^{-3}	—	0.54
,,	,,	1×10^{-3}	2.66
,,	,,	3×10^{-3}	3.33
	—	—	5.00
KCN	2×10^{-3}	—	5.22
Semicarbazide	,,	—	5.20
o-Phenanthroline	1×10^{-3}	—	5.70
Sodium azide	,,	—	5.40
Ethylene diamine tetracetate	,,	—	5.20

rather high concentration of pyridoxal phosphate (Table II). Other carbonyl reagents and metal chelators were without effect.

DISCUSSION

Since the discovery of alanine racemase in 1951 (*4*), there have been reports of a number of racemases, such as those for glutamic acid (*5*, *6*), threonine (*7*), methionine (*8*), proline (*9*), and diamino pimelic acid (*10*). Lysine racemase has also recently been isolated from *Proteus vulgaris* (*2*). Among these racemases, pyridoxal phosphate has been shown to be a cofactor for alanine and methionine racemase only. With the other racemases mentioned above, it was without effect. DPN has been reported to stimulate proline racemase and ATP and AMP to stimulate threonine racemase.

With the lysine racemase reported in this paper, pyridoxal phosphate had no effect. However since the inhibition caused by hydroxylamine could be reversed by pyridoxal phosphate it seems very likely that it is a cofactor.

An extract of an acetone powder of *Pseudomonas* cells which had been adapted to L-lysine has a very strong L-lysine oxidase, as well as a lysine racemase activity. This L-lysine oxidase has a very specific substrate affinity for L-lysine and is not inhibited by hydroxylamine (*11*). It is therefore possible to use this acetone powder for the differential determination of L- and D-lysine (*12*).

SUMMARY

1. Lysine racemase was isolated from *Pseudomonas* bacteria and purified.
2. The characteristics of this enzyme, such as the optimal pH and substrate concentration were examined.
3. The enzymatic neutralization of the optical rotation of the isomers of lysine was determined.

The authors are deeply indepted to Dr. Y. Izumi of the Department of Organic Chemistry of this Institute for his continuous interest and advice.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

STUDIES ON RIBONUCLEASES IN TAKADIASTASE

V. SYNTHETIC REACTION BY RIBONUCLEASE T_1*

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(Received for publication, March 28, 1960)

In this series of studies the preparation and specificity of RNase T_1*** and RNase T_2 have been already reported (*1–4*). RNase T_1 splits the secondary phosphate ester bond of guanosine-3′ phoshate in yeast RNA, via guanosine-2′,3′ cyclic phosphate. The mechanism of action of RNase T_1 is divided into two steps as in the case of RNase I, transphosphorylation and hydrolysis of phosphodiester. The reversibility of the former reaction by RNase I has been proved by Markham *et al.* (*5*). In this paper the synthetic action of RNase T_1 will be reported. It was proved that RNase T_1 could transfer guanylyl residue from $G_{cyclic}p$ to mononucleosides, mononucleotides, and monocyclic nucleotides producing respective dinucleoside monopnosphates and dinucleotides. Without additional guanylyl acceptors RNase T_1 synthesized oligo- or polyguanylic acids from guanosine-2′,3′ cyclic phosphate.

MATERIALS

RNA—Commercial yeast RNA (Schwarz) was deproteinized and reprecipitated with alcohol before use.

Cyclic Nucleotides—$G_{cyclic}p$ was prepared, as described in the previous report (*6*), from the dialysate of digestion mixture of RNA by RNase T_1. Pyrimidine nucleoside-2′, 3′ cyclic phosphates were isolated from the digestion of RNase T_1 core by RNase I as follows: A solution of RNase T_1 core (100 mg./2 ml.) was, after adjusting pH to 7.5, incubated with 10–20 μg. of RNase I at 37° for 2 hours and from the reaction mixture the 2′,3′ phosphates of cytidine and uridine were isolated by paper chromatography according to the method of Markham and Smith (*7*). Adenosine-2′,3′ cyclic phosphate was synthesized chemically by the method of Brown *et al.* (*8*).

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* The preliminary report of this study has been published in *Biochim. et Biophys. Acta*, 29, 655 (1958)

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*** Following abbreviations are used: RNase: ribonuclease, RNA: ribonucleic acid, 3′-Gp: guanosine-3′ phosphate, 3′-Ap: adenosine-3′ phosphate, 3′-Up: uridine-3′ phosphate, 3′-Cp: cytidine-3′ phosphate, $G_{cyclic}p$: guanosine-2′,3′ cyclic phosphate, $A_{cyclic}p$: adenosine-2′,3′ cyclic phosphate, $U_{cyclic}p$: uridine-2′,3′ cyclic phosphate, $C_{cyclic}p$: cytidine-2′, 3′ cyclic phosphate, the abbreviations of polynucleotides conformed to that of *Markham et al.* (*5*).

Mononucleosides ana Mononucleotides—They were commercial preparations and were proved to be pure by paper chromatography.

ECTEOLA-cellulose—ECTEOLA-cellulose was prepared by the method of Peterson and Sober (*9*) using Whatman cellulose powder.

RNase T_1—RNase T_1 was purified from "Takadiastase Sankyo" by heat treatment, ammonium sulfate fractionation, adsorption on and desorption from calcium phosphate gel, and Norit treatment. The preparation was subjected to zone electrophoresis under the condition described in the previous report (*1*), and the pattern of RNase T_1 was eluted and used in the following experiments.

Phosphomonoesterase—Phosphomonoesterase used in these experiments was prepared from human prostate by the method of Schmidt *et al.* (*10*).

RNase I—Commercial crystalline pancreatic ribonuclease was used.

METHODS

Paper Chromatographic Solvent—Solvent 1 : *Iso*-propanol-water (7 : 3 v/v) with ammonia in the vapour phase (0.45 ml. of concentrated ammonia water/liter of chromatographic chamber) (*7*). Solvent 2 : 70 ml. of tertiary butanol, 13.2 ml. of constant boiling HCl and water to 100 ml. (*11*). Paper used was Tôyôroshi No. 51 and No 51a.

Paper Electrophoresis—Electrophoresis was performed at 2° in 0.02 *M* citrate buffer at pH 3.5 in a field of 4 volts per cm. for 15 hours. Paper used was Tôyôroshi No 51a (15 cm × 40 cm).

General Procedure for Experiments—Reaction mixture containing RNase T_1, $G_{cyclic}p$ as guanylyl donor and desired acceptor in 0.1 ml. of 0.01 *M* phosphate bunffer (pH 7.0) was incubated. Aliquot of the reaction mixture was chromatographed on paper using solvent 1, or ECTEOLA-cellulose column as will be described later. In the paper chromatography substances produced by the synthetic action of RNase T_1 were detected on paper by the method described in the previous reports. For the identification of these substances chemical hydrolysis and enzymatic hydrolysis were carried out after eluting, and the products were determined by paper chromatography. For quantitative analysis the relative proportion of the guanine-content in the substances was determined after eluting the spots in 0.01 *N* HCl and reading the optical density at 255 mμ.

ECTEOLA-cellulose Column Chromatography—ECTEOLA-cellulose prepared was bufferized at pH 7.0 with 0.01 *M* phosphate buffer. After the reaction mixture had been incubated for the desired period it was subjected on the column. The elution was carried out by stepwise increase of the concentration of NaCl in 0.01 *M* phosphate buffer at pH 7.0. Identification of each fraction was carried out as follows: Dialysable fractions were subjected to Norit column and eluted from it with 50 per cent ethanol adjusted to pH 9 with ammonia. The eluate was concentrated after neutralization with formic acid and used for identification. Nondialysable fractions were subjected to identification after complete dialysis against distilled water.

Hydrolysis for Identification—For base analysis nucleotides were hydrolysed in 1 *N* HCl at 100° for 1 hour and purine bases and pyrimidine nucleotides produced were separated on paper chromatography using solvent 2. Or they were hydrolysed in 1 *N* NaOH at 37° for 18 hours and mononucleotides produced were identified with paper chromatography using solvent 1. Hydrolysis of cyclic phosphates was carried out in 0.1 *N* HCl at 2° for 18 hours (*2*). PMase treatment for splitting the terminal phosphomonoester group was carried out at pH 5.5. RNase T_1 was also used for hydrolysis at 37°, pH 7.5.

RESULTS

Transfer of Guanylyl Residue Catalysed by RNase T_1—When $G_{cyclic}p$ excess of uridine and RNase T_1 were incubated together and the reaction mixture was chromatographed, as shown in Fig. 1, another substance in addition

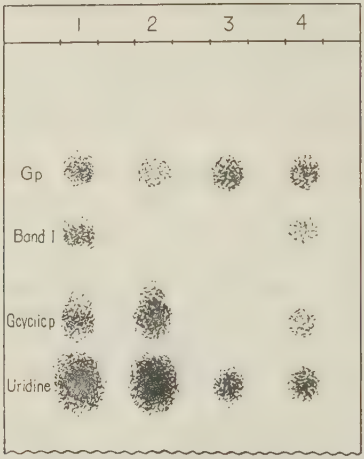

Fig. 1. Guanylyl transfer reaction from $G_{cyclic}p$ by RNase T_1. Col. 1, complete system. Reaction mixture contained 2.5 μmoles of $G_{cyclic}p$, 25 μmoles of uridine and 0.1 μg. of RNase T_1 in 0.1 ml. of 0.01 *M* phosphate buffer (pH=7.0) was incubated at 2° for 24 hours. Col. 2, no enzyme. Col. 3, eluate from band 1 was hydrolyzed by *N* NaOH at 37° for 20 hours. Col. 4, eluate from band 1 was incubated with RNase T_1 at 37° for 24 hours. Descending chromatogram ran in solvent 1 for 16 hours. Paper used was Tôyôroshi No. 51.

to $G_{cyclic}p$, 3′-Gp and uridine appeared (Band 1). This substance in band 1 gave by alkaline hydrolysis a guanylic acid and uridine and by RNase T_1 digestion gave a 3′-guanylic acid, $G_{cyclic}p$ and uridine. Consequently it was identified as guanylyl uridine. According to the changes of the concentration of uridine, incubation hours and temperature, the amounts of guanylyl uridine were altered and in some cases a substance which was not developed in the chromatographic solvent was detected. By alkaline hydrolysis of the substance, Gp and small amount of uridine were produced and the ratio of Gp to uridine was about 6–7. So it may be regarded to have a structure GpGp---U. These results are shown in Fig. 2 a, b, c, d. The production of guanylyl uridine increased in low reaction temperature and high concentration of acceptor. In the case of Fig. 2 d guanylyl uridine was formed in rather small amount and a large amount of nondeveloping substance appeared.

Specificity of RNase T_1 for Synthetic Reaction—As acceptors of guanylyl group, adenosine, guanosine, cytidine, uridine, adenylic acid, guanylic acid,

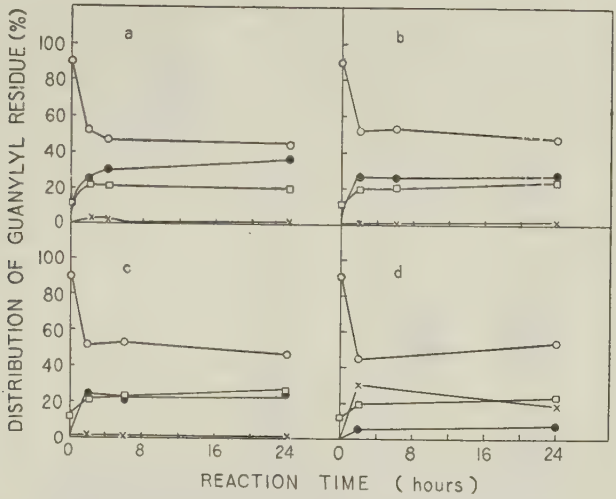

FIG. 2. Guanylyl transfer reaction from $G_{cyclic}p$ by RNase T_1. Each reaction mixture contained 2.5 μmoles of $G_{cyclic}p$, 0.1 μg. of RNase T_1 and uridine in 0.1 ml. of 0.01 *M* phosphate buffer (pH—7.0). In a, b, and c. uridine were 25 μmoles and in d. 5 μmoles. Reaction temperatures a.: 2°, b.: 24°, c.: 37°, d.: 2°. Abscissaes: reaction times, ordinates: per cent distribution of guanylyl residue measured by optical density at 255 mμ. —○—: $G_{cyclic}p$, —●—: band 1 in Fig. 1 Col. 1, —□—: 3′-Gp, —×—: nondeveloping substances (GpGp---U).

cytidylic acid, uridylic acid, 2′:3′ cyclic phosphate of adenosine, guanosine, cytidine and uridine were almost equally active and corresponding dinucleotides or dinucleoside monophosphates were produced. $A_{cyclic}p$ $U_{cyclic}p$ and $C_{cyclic}p$ could not behave as a donor of nucleotidyl residue in the synthetic reaction by RNase T_1.

Synthesis of Polyguanylic Acid—When a high concentrations of $G_{cyclic}p$ was incubated with RNase T_1 without additional guanylyl acceptor, several bands appeared in addition to $G_{cyclic}p$ and guanylic acid on paper chromatogram (Fig. 3 a). Band 1, band 2 and band 3 seemed to be $GpG_{cyclic}p$, GpGp and more polymerized guanylic acid respectively according to their R_f value. The nondeveloping substance (band 3) was subjected to the gallocyanine test which is positive on a poly- or oligonucleotides larger than tetranucleotide. (*12*). The result was positive and consequently the fraction contained such large polymerized nucleotides. The time course of the formation of these synthetic products was persued (Fig. 4). In the reaction mixture for 115 hours 38 per cent of the total guanylic acid was in the nondeveloping substances. 3′-Guanylic acid which was produced by the hydrolysis of $G_{cyclic}p$ by RNase T_1 decreased gradually for long incubation.

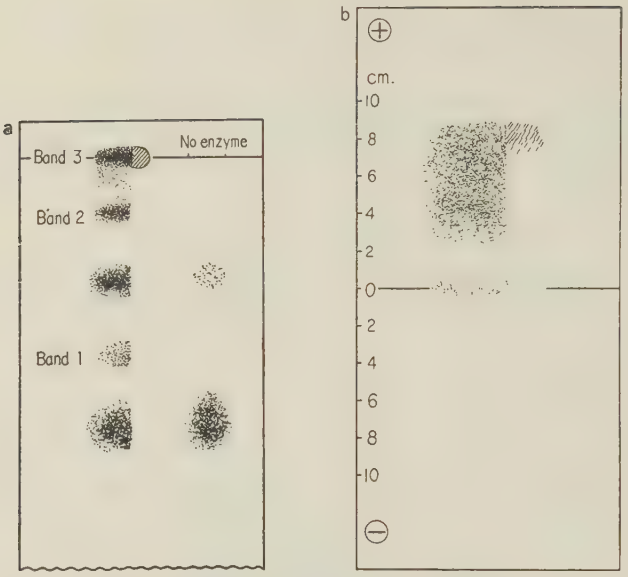

FIG. 3. Polyguanylic acid synthesis from $G_{cyclic}p$ by RNase T_1. Reaction mixture contained 20 μmoles of $G_{cyclic}p$ and 10 μg. of RNase T_1 in 0.1 ml. of 0.01 *M* phosphate buffer (pH 7.0) is incubated at 2° for 24 hours. a. Paper chromatogram using solvent 1. Band 1: $GpG_{cyclic}p$, Band 2: GpGp, band 3: oligo- or poly-guanylic acid. b. Paper electrophoresis. Electrophoresis was performed at 2° in 0.02 *M* citrate buffer at pH 3.5 in a field of 4 volt./cm. for 15 hours. Paper used was Tôyôroshi No. 51 a (15 cm. × 40 cm.).

: localized by U. V. absorption

: localized by gallocyanine reagent.

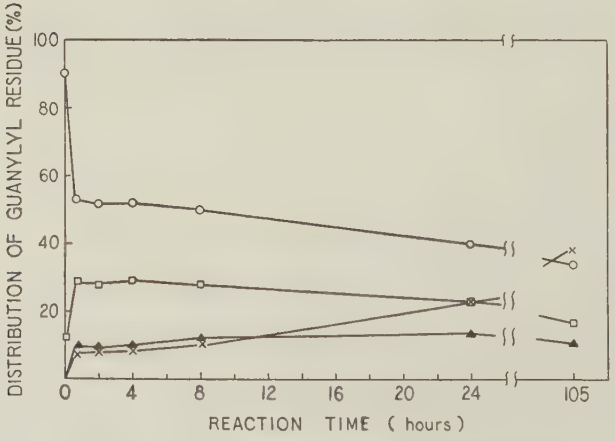

FIG. 4. Polyguanylic acid synthesis from $G_{cyclic}p$ by RNase T_1. Reaction mixture was the same as in the case of Fig. 3. —○—: $G_{cyclic}p$, —□—: 3′-Gp, —▲—: band 2 in Fig. 3 a. (GpGp), —×—: band 3 in Fig. 3 a. (GpGp--Gp).

For the purpose of separation of these polyguanylic acids the same reaction mixture was subjected to paper electrophoresis. The gallocyanine positive substances moved ahead of the bands of nucleotides (Fig. 3 b), but fractionation was not effected. Then ECTEOLA-cellulose column chromatography was carried out. Reaction mixture was adsorbed to the column in 0.01 *M* phosphate buffer (incubating condition) and eluted by the stepwise increase of concentration of sodium chloride. The elution curve is shown in Fig. 5. The eluate from 0.01 *M* phosphate buffer was mainly $G_{cyclic}p$ and

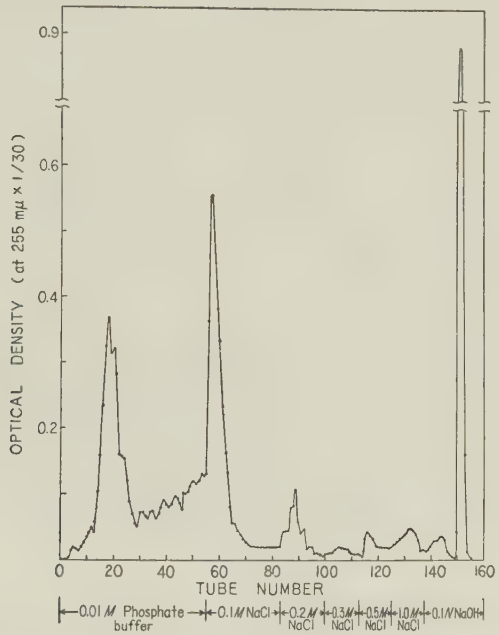

FIG. 5. Fractionation of polyguanylic acid synthesized from $G_{cyclic}p$ by RNase T_1 on ECTEOLA-Cellulose column.

Starting reaction mixture contained 100 μmoles of $G_{cyclic}p$ and 40 μg. of RNase T_1 in 0.5 ml. of 0.01 *M* phosphate buffer (pH=7.0) was incubated at 2° for one week in the presence of chloroform. Column was bufferized with 0.01 *M* phosphate buffer (pH=7.0). Eluting reagents are: 0.1 *M* (fraction 1), 0.2 *M* (fraction 2 and 3), 0.3 *M*, 0.5 *M* (fraction 4) and 1.0 *M* (fraction 5) NaCl in the buffer and finally 0.1 *N* NaOH (fraction 6 and 7). The pH of fraction 6 was neutral but that of fraction 7 was 8–9, so the fraction was neutralized by HCl as soon as possible. Flow rate was 12 ml./hour, and each tube was 4 ml.

followed perhaps by $GpG_{cyclic}p$, Gp and GpGp, but these were not separated. When eluate with 0.1 *M* NaCl (Fraction 1) was dialysed, about half of this fraction was dialysable and the fraction was positive to gallocyanine test. Eluates with higher concentration of NaCl were all nondialysable

against water, and ultraviolet absorption spectra of them were all that of guanylic acid. So these fractions seem to be four or more polymerized guanylic acid of various length. The yield from Fraction 7, containing perhaps the most highly polymerized guanylic acid, was about 2 mg.

Trial of Synthesis of Mixed Polymer by RNase T_1 and RNase I—RNase I had been shown by Markham *et al.* to catalyse the synthetic reaction from pyrimidine cyclic nucleotides. The auther tried to synthesize the polynucleotide containing guanylic acid and pyrimidine nuclectides from respective cyclic nucleotides by RNase T_1 and RNase I. The incubating condition was the best one for the synthesis of oligopyrimidine nucleotides from cyclic pyrimidine nucleotides by RNase I described by Markham *et al.* Namely reaction mixture which contained 40 mg. of cyclic guanylic acid, 15 mg. of cyclic cytidylic acid and cyclic uridylic acid, 10 μg. of RNase T_1 and 10 μg. of RNase I was incubated at 2° for 24 hours. Then the reaction mixture was subjected to ECTEOLA-cellulose column chromatography. However, the yield of reaction product was very low and the substances which were eluted with 0.1 *M* and higher concentration of NaCl did not contain any pyrimidine nucleotide.

DISCUSSION

It has already been reported by Markham *et al.* that RNase I catalyzed the synthetic reaction from pyrimidine nucleoside-2',3' cyclic phosphate. As the results of the experiments described above it was shown that RNase T_1 catalyzed also the synthetic reaction from $G_{cyclic}p$. In the reaction guanylyl residue acceptors were four nucleosides, nucleotides and cyclic nucleotides. These reactions may be shown as follows:

$$G_{cyclic}p+A = GpA$$
$$G_{cyclic}p+G = GpG$$
$$G_{cyclic}p+C = GpC$$
$$G_{cyclic}p+U = GpU$$
$$G_{cyclic}p+Ap = GpAp$$
$$G_{cyclic}p+Gp = GpGp$$
$$G_{cyclic}p+Cp = GpCp$$
$$G_{cyclic}p+Up = GpUp$$
$$G_{cyclic}p+A_{cyclic}p = GpA_{cyclic}p$$
$$G_{cyclic}p+G_{cyclic}p = GpG_{cyclic}p$$
$$G_{cyclic}p+C_{cyclic}p = GpC_{cyclic}p$$
$$G_{cyclic}p+U_{cyclic}p = GpU_{cyclic}p$$

In the case of RNase I pyrimidine nucleotidyl acceptors were only nucleosides and cyclic nucleotides, so RNase T_1 seems to have more broad ability for synthetic reaction than RNase I. As the difference from RNase I, in appropriate conditions RNase T_1 produced rather much amounts of tetra- or larger nucleotides with uridine as the acceptor. In addition to the fact that RNase T_1 could synthesized the oligo- or polyguanylic acid from $G_{cyclic}p$ these products were nondialysable and could be fractionated on the

ECTEOLA-cellulose column chromatography. The number of nucleotide units of each fraction has not yet been determined but considering the concentration of eluting NaCl these nucleotides may be rather large polymerized nucleotides. The studies on this point are now in progress.

In the experiment described above it was not successful to synthesize polynucelotide containing both pyrimidine nucleotide and guanylic acid by two RNases, RNase I and RNase T_1. But in a living cell it may be possible that these RNases synthesize the specific oligo- or polynucleotides or they involve transnucleotidation among some polynucleotides synthesized by other enzyme, for example, polynucleotidephosphorylase.

It is very interesting that by RNase T_1 rather large nucleotides were synthesized, and the studies on the chemical and physical properties of the oligo- or polyguanylic acids will be performed in future.

SUMMARY

1. It was proved that RNase T_1 catalyzed the transfer reaction of guanylyl residue from guanosine-2',3' cyclic phosphate to nucleosides and nucleotides.

2. RNase T_1 could synthesize the tetra- or higher polymerized guanylic acid from guanosine-2',3' cyclic phosphate.

3. These polyguanylic acids synthesized by RNase T_1 were fractionated on ECTEOLA-cellulose column.

The auther wishes to thank Prof. F. Egami for his helpful discussion and encouragement. She is indebted to Sankyo Pharmaceutical Co. Ltd. for the gift of "Takadiastase Sankyo", and to Dr. E. Iwase for the gift of U. V. filter of Scientific Researco Institute.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

ADDENDUM

INDUCTION OF STREPTOLYSIN S' FORMATION BY OLIGOGUANYLIC ACIDS

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(Received for publication, March 25, 1960)

The nature of active substance effective in the streptolysin S' formation by *Streptococcus pyogenes* has been studied in our laboratories, partly in collaboration with S. Ochoa, A. W. Bernheimer and their colleagues. High streptolysin-inducing activity was found in relatively large oligoribonucleotides rich in guanylyl residues (*1*). Moreover the studies (*2*, *3*) on the activity of polyribonucleotides synthesized by polynucleotide phosphorylase and the finding (*1*) that the terminal pyrimidine nucleotide of pancreatic ribonuclease core was not indispensable for the activity lead us to consider that the activity depends on the sequence of probably four or more guanylyl residues.

The present addendum to the preceding paper (*4*) deals with the induction of streptolysin S'-formation by oligoguanylic acids synthesized from guanosine 2', 3'-cyclic phosphate by ribonuclease T_1.

EXPERIMENTAL

Oligoguanylic acid: Oligoguanylic acids used are those eluted from Ecteola-cellulose column as illustrated in Fig. 5 of the preceding paper (*4*).

Determination of streptolysin S'-inducing activity and optical density: A proper volume of the mixture of 0.03 *M* $Na_2HPO_4 \cdot 12H_2O$, 0.03 *M* KH_2PO_4, 0.002 *M* $MgSO_4 \cdot 7H_2O$ and 0.01 *M* maltose was poured into the ampoule containing each lyophilized oligoguanylic acid sample so as to make the concentration ca. 400 μg. per ml. The quantity of each lyophilized sample in the ampoule had been estimated by measuring the optical density of each solution before lyophilization. Streptococcal cells obtained from 220 ml. of 7 hour-broth culture of *Streptococcus pyogenes*, S8, by centrifugation were washed twice with *M*/30 phosphate-buffered saline and suspended in 6.0 ml. of the same buffered saline. A half ml. of the cell-suspension was added to 0.5 ml. of each solution of oligoguanylic acid sample. The mixture was divided into three small test tubes which were then incubated at 37° in the water-bath for the formation of streptolysin S'. After the incubation respectively for 15, 45 and 90 minutes each tube was taken out, chilled immediately at 0° and then centrifuged at 3500 r.p.m. for 15 minutes. The sedimented cells were

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discarded and the clear supernatant was immediately put to test for hemolytic activity. The method of titration for hemolytic unit was exactly the same as described in the previous papers (*5*, *6*). The rest of each oligoguanylic acid solution was diluted 12- to 20-fold with distilled water and the optical densities for $\lambda=250\,m\mu$ and $\lambda=260\,m\mu$ of each diluted solution were measured. From the value thus obtained the optical density of the original solution was calculated.

RESULTS

As shown in Table I, the activity was observed, as expected, with relatively large oligoguanylic acids, especially with those eluted with 0.1 *N* NaOH.

Table I

Streptolysin S-inducing Activity of Various Fraction of Oligoguanylic Acid

Fraction No. of oligoguanylic acid	Hemolytic unit			Optical density[1]	
	15′	45′	90′	260 mμ	250 mμ
Fraction 1	0	0	0	5.34	5.17
Fraction 2[2]	—	—	—	—	—
Fraction 3	0	<1	4.2	3.06	2.80
Fraction 4	0	0	4.4	3.42	3.40
Fraction 5	0	ca. 1	4.0	4.80	4.78
Fraction 6	0	ca. 2	6.7	4.54	4.26
Fraction 7	33.2	35.2	24.4	4.50	4.41
Fraction 8	18.2	22.8	8.5	5.51	5.46

Fract. 1 : eluted with 0.1 *M* NaCl, Fract. 2, 3 : eluted with 0.2 *M* NaCl,
Fract. 4 : eluted with 0.5 *M* NaCl, Fract. 5 : eluted with 1.0 *M* NaCl,
Fract. 6 : eluted with 0.1 *N* NaOH (Eluate was neutral),
Fract. 7 : eluted with 0.1 *N* NaOH (Eluate was pH 8–9, and immediately neutralized)
Fract. 8 : Fract. 7 treated with pancreatic RNase.

1) Optical density of each solution used for the formation of streptolysin S.

2) could not be tested.

DISCUSSION

Streptolysin S′-inducing activity was observed with relatively large oligoguanylic acids. However the activity was found to be lower than that observed with pancreatic ribonuclease core from yeast RNA. The reason remains to be elucidated, however it might be explained at least partly by the competitive inhibition caused by smaller inactive oligoguanylic acids (*7*), which contaminate the active larger oligoguanylic acids.

SUMMARY

Large oligoguanylic acids enzymatically synthesized from guanosine 2′, 3′-cyclic phosphate were found to be effective for the induction of streptolysin S′-formation by *Streptococcus pyogenes*, S8.

The expense of this study was defrayed in part by a grant from the Ministry of Education.

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The Journal of Biochemistry, Vol. 48, No. 2, 1960

STUDIES ON THE CATALASE OF A THERMOPHILIC BACTERIUM

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(Received for publication, March 29, 1960)

Investigations of chemical nature of the enzymes contained in thermophilic organisms are of special interest from the view point of enzymology as well as of protein chemistry, because they are capable of functioning at extraordinary high temperatures where most of the ordinary proteins and enzymes lose their functions owing to the denaturation caused by heat. Militzer and his coworkers (*1–8*) found that various enzymes contained in thermophilic bacteria (malic dehydrogenase, adenosine triphosphatase, apyrase and pyrophosphatase) have their temperature optima in the range between 60° and 65°, where also the organisms show optimum growth activity. That the amylase of thermophilic bacteria has a remarkable stability against heat has been observed by several workers (*9*, *10*).

The present study deals specifically with the nature of a catalase isolated from a thermophilic bacterium, which was found to show a characteristic phenomenon of heat-activation upon transference from ordinary temperature to high temperatures. It was revealed that a crude preparation of the enzyme isolated from bacterial cells contains a specific component(s) which by combining reversibly with catalase molecule suppresses the activity of the latter. The activation caused by heat was attributed to the thermal dissociation of the component from the enzyme. In this paper are described the method of separation of the suppressing factor from the enzyme and some experimental results dealing with the interaction of the factor and enzyme, which seems to throw some light on the mechanism of heat-activation phenomena observed in general in the physiology of thermophilic organisms.

MATERIAL AND METHOD

Material—The organism used as material was a thermophilic bacterium which was isolated from soil in our institute. The growth characteristics of the organism at various temperatures have been studied by Hirano *et al.* (*11*), who found the optimal temperature for growth to be 65°. The medium used for the culture had the following composition: peptone 2.5 per cent, K_2HPO_4 0.3 per cent, $(NH_4)_2SO_4$ 0.13 per cent, NaCl 0.05 per cent, $MgSO_4 \cdot 7H_2O$ 0.025 per cent, $FeSO_4 \cdot 7H_2O$ 0.002 per cent and agar 3 per cent. The pH of the medium was adjusted so as to obtain a solution of pH 7.0–7.2 after autoclaving. The bacterium was inoculated to 800 ml. of this medium in a shallow tray

and was incubated at 65°. After 20–24 hours of culture, the bacterial cells were harvested, washed with 0.01 *M* phosphate buffer of pH 7.0, resuspended in the same solution, and then used as the material for the preparation of the cell-free catalase solution.

Preparation of Crude Catalase Solution—A small amount of crystalline lysozyme (*12*) prepared from egg white was added to the washed bacterial suspension, and the mixture was incubated at 35°–40° for 2–3 hours, being shaken continuously. A yellow transparent supernatant obtained by centrifuging the viscous lysate at $100{,}000 \times g$ for 20 minutes was used as the "crude" catalase sample. The sample thus obtained, which contained, besides catalase, a "suppressing factor" (S-factor) acting upon it, showed absorption bands at 630–650, 540–550, 480–490 and 406 mμ*. The positions of the bands except for that of the Soret band seem to be considerably different from those found for ordinary preparation of catalase.

Preparation of Catalase Free from "Suppressing Factor"—To 10 ml. of the crude enzyme solution obtained as above, 0.8 g. of charcoal powder was added, and the mixture was kept at 65° for 10 minutes. The charcoal, to which the S-factor was adsorbed, was filtered off, and the clear filtrate was used as the preparation of "S-free" catalase.

Preparation of S-Factor Free from Catalase—The S-factor free from catalase was obtained by boiling the crude catalase solution at 100° for a few minutes. A transparent supernatant obtained by centrifuging the boiled solution was used as the preparation of the S-factor free from catalase.

Measurement of Catalase Activity—The course of H_2O_2-decomposition effected by catalase was followed either by ordinary titrimetric method using potassium permanganate (0.01*N*) or by a spectrophotometric method measuring the decay of the absorption at 240 mμ shown by H_2O_2. The latter method, which proved to be more accurate than the other, was performed using a Cary Recording Spectrophotometer, Model 14 *M*, by the following procedure. The solution of catalase (either crude or S-free) which had been prepared as described above was diluted (in most cases 2–4 fold) so as to obtain adequate rate of H_2O_2-decomposition. The reaction mixture consisted of 1 ml. of the diluted enzyme solution, 13 ml. of phosphate buffer of pH 7.0 and 1 ml. of H_2O_2 solution (final concentrations: 0.02 *M* H_2O_2 and 0.033 *M* phosphate). The mixture was promptly poured into a 1 cm. cell, and the decay of the absorbance at 240 mμ was recorded against time, taking as a reference a mixture which contained distilled water instead of the H_2O_2 solution. The record of the reaction process could be started within 15 seconds after mixing the reaction components.

Titrimetric measurement was conducted in the experiments performed at 0°, since it was difficult to use the spectroscopic method at this temperature. The reaction mixture used in this case was essentially the same as above, the only modification being that the initial concentration of H_2O_2 applied was 0.01 *M*. At intervals of 20 seconds, aliquots (2.0 ml. each) of the mixture were pippetted into a dilute H_2SO_4 solution, and H_2O_2 was titrated with 0.01 *N* $KMnO_4$ solution.

As will be shown later, the time course of enzymatic H_2O_2-decomposition was modified in specific manners by the presence of the S-factor. Comparison of the activities of crude and S-free enzyme preparations under various experimental conditions was made by measuring in each case the *initial* velocity of H_2O_2-decomposition.

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* Exact positions of the bands other than that at 406 mμ could not be determined because of the low concentration of the enzyme contained in the sample.

RESULTS

1. General Properties of S-Free Thermophilic Catalase

Before presenting the data showing the effect of the S-factor upon the "S-free" thermophilic catalase, it seems pertinent to describe the general properties of the free enzyme as compared with those of ordinary catalase contained in such materials as mammalian liver, mesophilic microorganisms, *etc.*

The thermophilic catalase freed from the S- factor was found to share the following properties with the ordinary catalase:

i) At lower temperature it decomposes H_2O_2 according to the first-order rate law, and at higher temperatures the reaction deviates more or less from this law because of the partial destruction of enzyme caused by H_2O_2. It should, however, be noticed that both the lower and higher temperatures mentioned here are considerably higher than those for the ordinary catalase.

ii) At a given temperature, the rate of H_2O_2-decomposition is proportional to the concentration of the enzyme applied (see Fig. 2). As will be shown later, this fact contrasts greatly with the phenomenon observed with the crude enzyme preparation containing the S-factor.

The principal differnce between the S-free catalase and the ordinary ones lies in the remarkable thermophilic nature of the former (see the data presented in Fig. 3). Insofar as the activity was measured by the initial rate of H_2O_2-decomposition, the optimal temperature of the S-free enzyme was found to lie at 60°, and the enzyme activity did not disappear even at70°.

2. Effect of Suppressing Factor upon S-Free Catalase

When the S-free catalase was brought together with the S-factor, there occurred a gradual decrease of the enzyme activity. The suppression of the

TABLE I

Effect of Suppressing Factor upon "Free" Catalase

	Relative activity[1] of the enzyme	
	in the presence of S-factor	in the absence of S-factor
Immediately after mixing	1.00	1.00
After 24 hours' incubation at 0°	0.28	0.99
After 5-minutes' heating at 65° of the incubated mixture	0.86	0.99

1) In all cases, enzyme activity (initial rate of H_2O_2-decomposition) was measured at 0°. The reaction rate which was observed immediately after the preparation of the mixture was taken as 1.0 to express the relative activities.

enzyme activity was found to be more marked at lower temperatures, indicating that the S-factor formed with the enzyme a heat-dissociable inac-

tive complex. In the experiment presented in Table I, 2 ml. of the S-free enzyme preparation was mixed either with 2 ml. of a solution of the S-factor or 2 ml. of water (as a control). The enzyme activity (initial rate of H_2O_2-decomposition) was measured at 0°: (i) immediately after the preparation of the mixtures, and (ii) after 24 hours' incubation of the mixtures at 0°. The incubated mixtures were then heated at 65° for 5 minutes and, after lowering the temperature down to 0° (which took 5 minutes), the enzyme activities were also measured at 0°. As may be seen from the table, the enzyme activity in the presence of the S-factor was lowered markedly (to 28 per cent) after 24 hours' incubation at 0°, and this suppression was greatly recovered (to 86 per cent) by subsequent heating of the mixture at 65°. In contrast, the "free" catalase without added S-factor showed the same activity in the three measurements. More detailed data concerning the mode of interaction between the enzyme and the S-factor as it is influenced by temperature will be given later.

3. The Mode of Interaction between S-Factor and Catalase

To investigate the mode of interaction between the enzyme and the

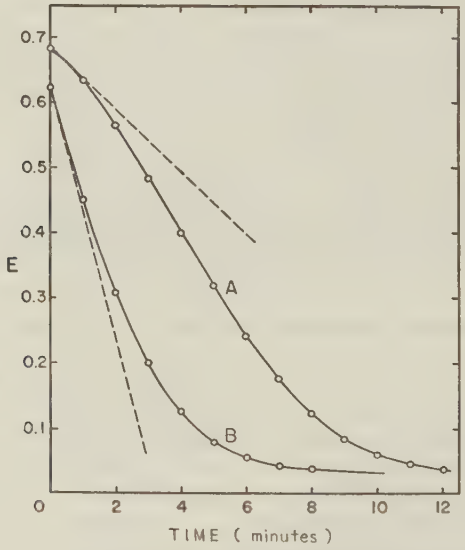

FIG. 1. Time courses of H_2O_2-decomposition caused by crude and S-free enzymes.

The concentration of H_2O_2 was followed by measuring the absorbance (E) of H_2O_2 at 240 mμ. Curve A: curde enzyme; Curve B: S-free enzyme.

S-factor, the behaviours of the enzyme in the crude sample (containing the S-factor) and in the "free" state were compared under various experimental conditions. The "free" enzyme used in each experiment was prepared by

treating with charcoal the crude sample used in the same experiment. The test samples used in each set of experiment may, therefore, be regarded as containing the same amount of the enzyme.

Time Course of H_2O_2-Decomposition—The process of H_2O_2-decomposition caused by the crude preparation of the enzyme followed a course which is somewhat different from that shown by "free" enzyme. In Fig. 1 are compared these two processes occurring at the temperature of 15°. While the "free" enzyme gave a curve which corresponded to a simple first-order reaction, the crude enzyme gave an S-shaped curve, showing that there occurred an increase of the reaction rate at the initial stage of the reaction. This increase in rate seemes to be due to the competitive action between the S-factor and H_2O_2 in their combination with the enzyme. The result presented in the figure also shows that the enzyme activity—when compared by the initial rate of H_2O_2-decomposition—was increased about 4-fold by the removal of the S-factor.

Effect of Dilution of Enzyme Solution—The solution of crude and S-free enzymes were diluted with phosphate buffer (pH 7.0) stepwise to obtain solutions having 1, 1/2, 1/4, 1/8 and 1/16 of the original concentrations. The samples of diluted solutions were kept at 20° for one hour, and their enzymatic activities were measured at a room temperature (18°–20°). The results obtained are presented in Fig. 2, in which the relative enzymatic

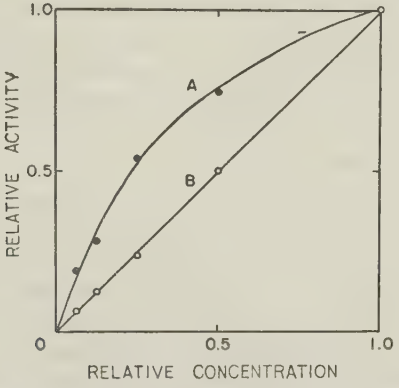

Fig. 2. Effect of dilution of enzyme solutions.
Curve A: crude enzyme; Curve B: S-free enzyme.

activities (the activity shown by the original solution being taken as unity) are plotted against relative concentrations of the solutions.

As is apparent from these results, the relative activities of the crude sample were markedly increased by the effect of dilution, while those of S-free enzyme preparation did not show the same phenomenon. The increase of relative activity of the crude sample was more pronounced in more diluted solutions. The highest relative increase, which was observed at 16-fold dilution, was as much as 3-fold. These results make it plausible to

assume that the combination between the enzyme and the S-factor, which are coexisting in the crude sample, follows the law of mass action.

Temperature Dependency of the Enzyme Reaction—The crude and S-free enzyme preparations were compared for their temperature dependency of enzyme activity. Temperatures applied ranged from 0° to 70°. The enzyme preparations were kept 10 minutes in a thermostat of a given temperature, and the rate of H_2O_2-decomposition was measured at the same temperature. The results obtained are summarized in Fig. 3, in which the relative enzyme activities are compared by taking the activity shown by crude enzyme sample at 0° as unity. In the figure, Curve A and B represent, respectively, the data obtained with the crude and S-free enzyme preparations, and Curve C shows the temperature dependency of the growth rate of the thermophilic bacterium used for the preparation of the enzyme*. The following facts emerge from these results.

i) The optimum temperature lies at 65° for the crude enzyme and at about 60° for the S-free enzyme. The ratio of the activities at these temperatures to those at 0° was 4 : 1 for the S-free enzyme and 40 : 1 for the crude enzyme. The apparent activation energy of the enzyme reaction, which was calculated from the data obtained between 35° and the optimum temperature was 3 ± 1 Kcal./mole** for the S-free enzyme and as much as 14 ± 1 Kcal./mole for the crude enzyme.

ii) In the temperature range between 0° and 55°, the activity of the S-free enzyme was higher than that of the crude enzyme, and the reverse was true at the temperature above 55°. In the temperature range below 55°, the ratio of the activity of S-free enzyme to that of the crude one increased considerably with the decrease of temperature. The highest ratio which was observed at 0° was 7.6 : 1.

iii) The optimum temperature for the crude enzyme coincided well with the peak of the temperatuse-growth curve obtained with the original thermophilic bacterium***.

The fact that in the temperature range below 55° the activity of the S-free enzyme was higher than that of the crude enzyme is obviously due to the absence of the S-factor in the "free" enzyme preparation. That the activity ratio of the two samples approached gradually to 1 with the increase of temperature, may be explained as being due to the situation that the combination between the enzyme and the S-factor was loosened with the increase of temperature. Considering this situation, we can also explain the fact that, in the suboptimum temperature range, the crude enzyme showed a considerably higher activation energy than S-free enzyme. Namely, the apparent activation energy shown by the crude

* Data reported by Hirano *et al.* (*11*)

** This value is of the same order of magnitude as the activation energy (1.7–4.3 Kcal./mole) determined by several workers for ordinary catalases.

*** Similar phenomena have been observed by Militzer *et al.* (*1–8*) for various enzymes contained in thermophilic bacteria.

enzyme was a combined result of two processes: the dissociation of the S-factor from the enzyme, and the activation of the enzyme reaction itself. A convincing evidence for this inference will be presented in a later section.

Attention should be called to the fact that at the temperatures above 60° the crude enzyme showed higher activities than the S-free enzyme, and that the optimum temperature of the latter was lower than that of the former. Decrease of the activity of the S-free enzyme at higher temperatures was apparently due to the inactivation of the enzyme caused by heat (in the presence of H_2O_2). The inactivation was so profound at 70° that a part of H_2O_2 added remained undecomposed in the reaction mixture, although the initial rate of the H_2O_2-decomposition was rather high. These observations make it plausible to assume that the procedure of charcoal treatment of the crude enzyme was effective in removing not only the S-factor, but

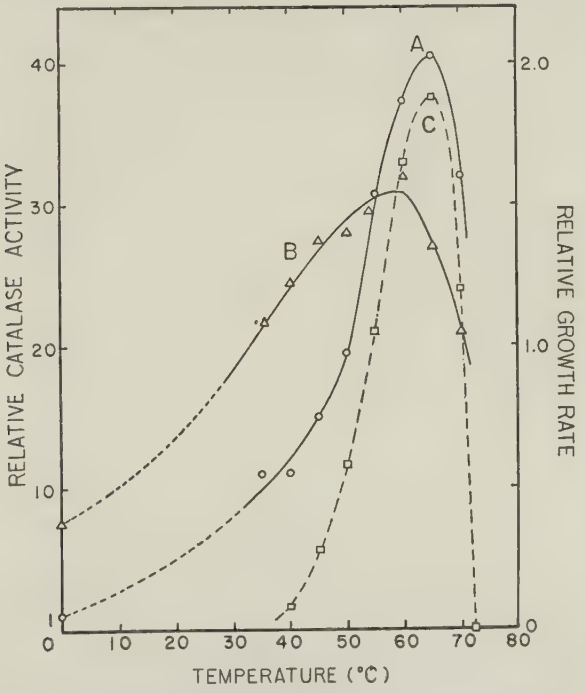

Fig. 3. Temperature dependency of the enzyme reaction as compared with that of the growth activity of the original thermophilic bacterium.

Curve A: crude enzyme; Curve B: S-free enzyme; Curve C: growth activity (measured at the logarithmic phase).

also a certain factor which protected the enzyme against the destruction caused by heat and/or H_2O_2. Whether this factor is the S-factor itself or something differnt from it is a question left open for further investigations.

Effect of Heat Treatment Followed by Cooling—Interesting phenomena were observed when the activity of the crude enzyme was measured at 0° after it had been subjected to pre-heating at a high temperature (65°) followed by cooling to 0°. The crude enzyme, whose activity had been measured at 0°, was heated and kept at 65° for 1 hour, and then cooled down to 0°. During the whole period, aliquots of the sample were taken out at intervals, and the activity was measured at 0°.* In Fig. 4, which reproduced the results obtained, the activities of the samples at different stages of the experiment are compared by taking the original activity (observed before the heat treatment) as unity. As is clear from the figure, the change of temperature from 0° to 65° caused a 15-fold increase** of the activity within 15 minutes and the activity remained in the same level at the same temperature. When the temprature was subsequently lowered to 0°, the activity decreased slowly, and it appeared that the activity eventually returned to the ooiginal level, although it was not quite the case even after 19 hours' incubation at 0°.

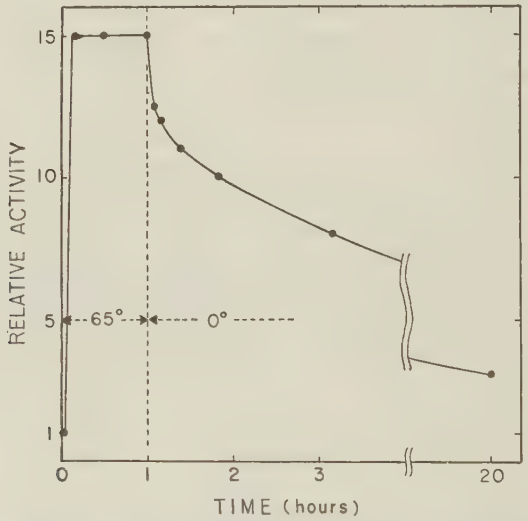

Fig. 4. Effect of heat-treatment (at 65°) followed by cooling upon the activity of the crude enzyme. From the enzyme solution kept at the temperature indicated in the figure, aliquots were taken out at intervals, promptly cooled down to 0°, and their enzymatic activities were measured at 0°.

Nevertheless, the activation by heat and deactivation by subsequent cooling could be repeated several times with one and the same sample, indicating

* It took approximately 5 minutes for cooling the heated samples to 0°.

** The degree of the activity increase caused by the same treatment varied considerably (in the range from 5- to 20-fold) according to the sample of the crude enzyme tested.

that the process is practically of a reversible nature.

When an experiment of the same arrangement was performed using the S-free enzyme, there occurred neither the activation by heat treatment nor the deactivation at 0° after the heat-treatment. We may, therefore, conclude that the characteristic change of the enzyme activity caused by heat-treatment (Fig. 4) was a result of an interaction between the enzyme and the S-factor contained in the crude sample.

Estimated Free Energy of Dissociation of S-Enzyme-Complex—Since the deactivation at 0° of the crude enzyme, which occurred after the heat pre-treatment, was a rather slow process, we may assume that the activity of the heat-treated crude enzyme, which was measured at 0° only 5 minutes after the sample had been cooled to this temperature, represents the activity at 0° of the sample in the heat-activated state before cooling. If this latter activity may be regarded as corresponding to the concentration of the enzyme, from which the S-factor had been dissociated by heat, we can investigate by the same method of measurement the relative degree of disso-

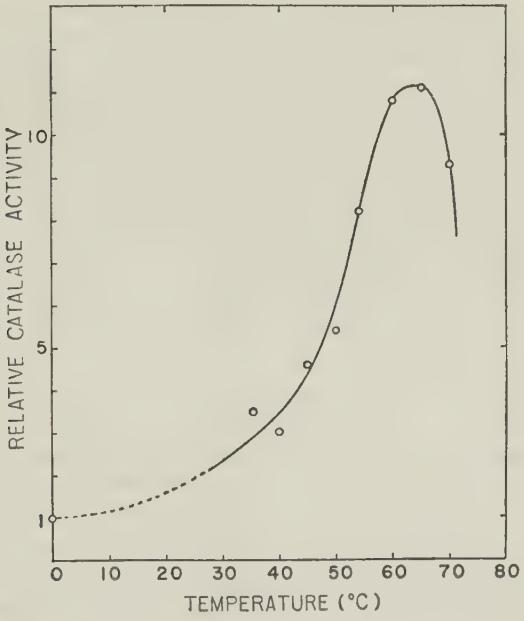

Fig. 5. Effect of incubation temperatures upon the activity (measured at 0°) of the crude enzyme. The enzyme sample tested was the same as that used in the experiment in Fig. 3. See text for full explanation.

ciation of the S-factor from the enzyme at different temperatures. In the experiment reproduced in Fig. 3, parallel measurements were made at 0° (5 minutes after cooling) of the activity of the crude enzyme, which had

been incubated for 10 minutes at different temperatures ranging from 0° to 70°. The results obtained are presented in Fig. 5, in which the relative activities measured are expressed by taking the activity (at 0°) of the non-heat-incubated crude sample as unity. As may be seen, the maximum activity was observed at 65° which also represented the optimum temperature for the crude enzyme activity measured at respective temperatures (Fig. 3). Using the values obtained between 40° and 60° the apparent activation energy was computed to be 12±1 Kcal./mole, which, on the basis of the assumption made above, may be regarded as being the free energy of the dissociation of the S-factor from the enzyme. As mentioned before, the "free" enzyme showed—in the temperature range between 35° and 60°—an activation energy of 3±1 Kcal./mole. The sum of this energy and the free energy of dissociation estimated here agree satisfactorily with the apparent activation energy (14±1 Kcal./mole) of the crude enzyme measured in the experiment presented in Fig. 3.

DISCUSSION

We believe to have shown that the crude catalase preparation obtained from the thermophilic bacterium contains a specific substance(s) acting suppressively upon the enzyme action, and that this substance dissociates from the enzyme not only on heat-treatment, but also on dilution of the reaction mixture. Existence of natural suppressors or inhibitors has been reported by many workers for various enzymes in various biological systems, such as catalase in normal or carcinomatous mammalian liver (Greenstein *et al.* (*13*), Nakahara *et al.* (*14*), Deutsch *et al.* (*15*, *16*), Ceriotti *et al.* (*17*), Alexander (*18*), Heim *et al.* (*19*, *20*), Sugimura (*21*), Margoliash *et al.* (*22*) and Feinstein *et al.* (*23*)), catalse of sea urchin egg during their development (Deutsch *et al.* (*24*)), catalase of silk worm (Ohoka*), pyrophosphatase in *Proteus vulgaris* (Swartz *et al.* (*25*)), *etc.* The modes of action of these suppressors are apparently different from case to case, but there are some cases which bear, at least phenomenalistically, certain resemblances to the phenomena observed in the present study. For instance, the inhibitor of catalase found by Deutsch *et al.* in tumor tissues and by Ohoka in silk worm has a dissociable nature which is affected by temperature in a manner similar to that observed in the present experiments.

The biological significance of these natural inhibitors may also be different from case to case. Swartz *et al.*, studying the natural inhibitor of pyrophosphatase in *Proteus vulgaris*, maintain that the bacterial cells contain various natural inhibitors for specific enzymes and that they would act as controllers of the metabolic patterns of bacterial cells under different conditions. The biological significance of the S-factor found in the present study is difficult to interpret, all the more because the significance of catalase in living organisms in general remains largely obscure at present. However,

* Ohoka, T., private communication.

the fact that the optimum temperoture of the thermophilic catalase (in the presence of the S-factor) coincides almost exactly with that of the bacterial growth may be regarded as evidence that this enzyme is playing some important role in the cellular activity of the organism. Hirano *et al.* (*11*) showed that the growth activity of the thermophilic bacterium, from which the enzyme investigated in the present study was isolated, was enhanced enormously when the temperature was raised from 15° or 30° to 65° for a short time. This phenomenon is quite similar to the remarkable heat-activation observed with the "crude" enzyme, indicating again the close parallelism existing between the enzyme activity and the growth activity of the organism.

The experiment now in progress in our laboratory showed that the S-factor also exerts an inhibitory effect upon ordinary bovine liver catalase. As mentioned already, there is an indication that the S-factor combines with the thermophilic catalase competitively with H_2O_2. The chemical nature of the S-factor as well as the precise mechanism of its suppressing effect upon catalase is a matter for further investigation. Side by side with these problems, there remains another important question regarding the cause of the thermostability of the enzymes contained in thermophilic organisms. As we have shown in our experiment (Fig. 3), the S-free catalase, which was by itself remarkably heat-stable compared with the ordinary catalase, showed a lower resistance to heat than did the crude enzyme. Presumably, the crude enzyme contained some factor protecting the enzyme against heat, and this factor might have been removed by the charcoal-treatment which was used for the separation of the S-factor from the enzyme. Further studies of the cause of heat-stability of thermophilic enzyme seem to involve two questions: one regarding the possible existence of a specific factor which protects the enzyme against heat, and the other regarding the physico-chemical cause of the thermostability of enzyme protein itself.

SUMMARY

1. From a lysate of a themophilic bacterium a cell-free solution was prepared, which contained a thermophilic catalase together with a certain factor(s) having a specific suppressing effect upon the catalase at lower temperatures. The catalase could be freed from the suppressing factor (S-factor) by charcoal-treatment of the solution, and the S-factor free from the enzyme was obtained by boiling the same solution. In the coexistence of these two components, the apparent catalytic activity of the solution changed in characteristic manners in response to the changes of temperature and of concentrations of the components in the reaction mixture. By quantitative studies of these phenomena it was concluded that tne S-factor combines reversibly with the enzyme to form a heat-dissociable inactive complex.

2. The optimum temperature of the "free" catalase lies at 60°, while it shifts to 65° in the presence of the S-factor. The latter value coincides

with the optimum temperature for the growth of the original thermophilic bacterium. The apparent activation energy of the enzyme reaction (in suboptimal temperature ranges) in the presence and absence of the S-factor was found to be about 14 and 3 Kcal./mole, respectively. The remarkably high value obtained in the presence of the S-factor was concluded to be a combined result of the dissociation of the S-factor from the enzyme and the activation of the enzyme reaction itself. Along the line of this inference and based on the results of experiments, in which the enzyme-S-factor-system had been pre-incubated at various temperatures and its enzyme activity was subsequently measured at 0°, it was deduced that the free energy of the dissociation of the inactive complex was 12 Kcal./mole.

3. The catalase freed from the S-factor behaved in respects of reaction kinetics—except for its remarkable temperature-tolerance—like ordinary catalase.

The author wishes to express his heartfelt gratitude to Prof. H. Tamiya, Prof. A. Takamiya, and Prof. K. Shibata for their guidance and valuable advices in carring out the experiments described here.

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The Journal of Biochemistry, Vol. 48, No.2, 1960

THE INCREASED URINARY EXCRETION OF L-ASCORBIC ACID IN ALLOXAN-DIABETIC RATS

Burns *et al.* (*1*) have reported that various barbiturates, other hypnotic drugs such as chloretone, and certain antipyretic and analgestic drugs increase the urinary excretion of L-ascorbic acid and D-glucuronic acid. In this communication, the urinary excretion of L-ascorbic acid in alloxan-diabetic rats is reported.

Male albino rats weighing 120 to 150 g. were maintained on a stock diet. Seven days after injection of 50 mg. per kg. of alloxan, diabetic rats which had a fasting blood sugar in excess of 200 mg. per 100 ml. were selected for the study. In insulin treatment 1 unit per kg. per day of lente insulin (Novo) was used. Barbital was given as sodium salt 300 mg. per kg. per day by intraperitoneal injection. L-Ascorbic acid excreted in the urine was determined by titration with indophenol dye (*2*) after the Dowex-1 (Cl^-form) treatment. The cytoplasmic suspension of rat liver was prepared by the procedure of Chaikoff *et al.* (*3*). Hepatic destruction of L-ascorbic acid was measured by the dinitrophenylhydrazine method of Roe and Kuether (*4*) with a modification.

TABLE I

Urinary Excretion and Hepatic Destruction of L-Ascorbic Acid in Control, Alloxan-Diabetic and Barbital-Treated Rats

Animal	No. of rats	Urinary L-ascorbic acid[1]	Hepatic destruction of L-ascorbic acid[2]
		mg. per day	mμmoles
Control	4	0.26±0.07	57.4±4.7
Alloxan-diabetic	6	0.59±0.13	35.3±3.1
Alloxan-diabetic (insulin-treated)	6	0.20±0.05	59.7±4.0
Barbital-treated	5	1.35±0.53	60.9±6.3
Barbital-treated (insulin-treated)	5	1.29±0.39	57.1±7.1

1) Urine samples were collected in 10 per cent oxalic acid. Average value for L-ascorbic acid excretion and standard errors are given in the table.

2) The reaction mixture contained 0.2 μmole of L-ascorbic acid, 50 μmoles of phosphate buffer, pH 7.0, 1.8 μmoles of DPN, 2.3 μmoles of ATP and 0.3 ml. of cytoplasmic suspension of rat liver in a total volume of 1.0 ml. After incubation for 2 hours at 37° L-ascorbic acid was measured by the method of Roe and Kuether with a modification.

The results are presented in Table I. The urinary L-ascorbic acid excre-

tion was increased in alloxan-diabetic rats and restored to normal by insulin treatment. However, in barbital-treated rats insulin had no effect on L-ascorbic acid excretion. The destruction of L-ascorbic acid was lowered in alloxan-diabetic rat liver extract. In barbital-treated rat liver extract the diminished L-ascorbic acid destruction was not observed.

These results suggest that the increased urinary excretion of L-ascorbic acid in alloxan-diabetic rats was partially the result of the lowered destruction of L-ascorbic acid in tissues caused by the insulin deficiency unlike barbiturates *etc.* However, the stimulatory effect on L-ascorbic acid synthesis may also be considered in alloxan-diabetic rats. Burns *et al.* (*1*) also have reported that the stimulatory effect on the glucuronic acid pathway by barbiturates *etc.* was considerably less in hypophysectomized rats than in normal rats, suggesting possible hormonal control on the glucuronic acid pathway. Experiments with hypophysectomized rats are also in progress in this laboratory.

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(Received for publication, May 6, 1960)

The Journal of Biochemistry, Vol. 48, No. 2, 1960

A FILAMENTOUS PROTEIN FROM THE CLEAR PHASE OF MYOSIN B

It has been known for some time that the solution of myosin B in 0.15 *M* KCl becomes less turbid upon the addition of adenosinetriphosphate (ATP) until the superprecipitation sets in. Spicer (*1*) named this phenomenon "the clear phase". When the solution of myosin B in the clear phase was investigated with the technique of flow birefringence, it was found (*2*) that all the constituent protein in the solution was dispersed in the form of fibrous molecules, *i.e.* the birefringence of the solution in the clear phase was 1.2×10^{-2} when reduced to unit orientation factor and unit concentration. From the extinction angle, it was also found (*2*) that the molecules in myosin B solution in the clear phase had almost uniform length of about one micron.

When ultracentrifugation pattern was examined for myosin B solution in the clear phase, three peaks were observed corresponding to sedimentation coefficient values of 7, 40 and 100 S. The latter two peaks were similar to the values for myosin B in 0.6 *M* KCl (*3*) and for myosin A in 0.2 *M* KCl (*4*) respectively, and the molecular configuration for them may similarly be assumed when considered together with the results of flow birefringence experiments. The peak at the sedimentation coefficient of 7 S, at first glance, seemed to be due to the presence of myosin A. The sedimentation coefficient for myosin A in 0.6 *M* KCl is about 7 S (*3*, *5*, *6*). However, the flow birefringence result mentioned above required that this component should also contribute together with the other two components to the birefringence of the solution in the clear phase, by being aligned to a considerable extent at the flow gradient of about 1000 sec^{-1}. The amount of the slowest sedimenting fraction was estimated by the Rayleigh refractometric method to be about 1/4 of the original protein concentration, and the presence of the slowest component could not be neglected. Therefore, it was presumed that the slowest sedimenting component had a length comparable to those of two other components, though it should be very narrow in width in order to account for the slow sedimentation coefficient of 7 S.

The solution of myosin B from rabbit skeletal muscle in 0.15 *M* KCl was centrifuged at $100{,}000\times g$ for two hours in the presence of 5 m*M* each of ATP and $MgCl_2$, and a water clear supernatant solution was obtained. This supernatant solution contained about 1/5 to 1/4 of the initial protein, and showed flow birefringence. The extinction angle indicated that the length of molecules was a few micra, and there was a tendency that the

extinction angle to increase, *i.e.* the length to decrease, after the solution was subjected to a shear rate of higher than 1000 sec.$^{-1}$ for several minutes. When ATP was removed from this supernatant solution by dialysing over night, the solution showed no apparent change. However, when examined in the flow birefringence apparatus, it showed birefringence only for a very short period, and then the solution became turbid. The reason for this seemed to be that the molecules aggregated due to the application of a shearing field. Numerous hardly visible particles appeared in the field of the flow birefringence apparatus. Similar kind or particles were observed (*7*) after the addition of ATP to myosin B solution which had not been clarified by centrifugation.

When the ionic strength of the supernatant solution was raised by the addition of KCl, the extinction angle increased and approached 45°, and the birefringence decreased, indicating the dissociation of the long molecules into shorter ones. In solutions of KCl of the concentration higher than 0.4 *M*, the dissociation was almost complete and the rotary diffusion coefficient calculated from the extinction angle was about 1000 sec.$^{-1}$

In the supernatant solution obtained from the clear phase, no superprecipitation took place, even when some myosin A was added. No sign of complex formation was observed after the addition of myosin A. When the amino acid composition of the protein in the supernatant was analyzed, the ratio of the proline content to arginine content was Pro. Arg.=0.92, while the ratio is 1.15 for action and 0.54 for myosin A (*8*). The supernatant solution in the presence of Mg^{++} and ethylenediaminetetraacetate at pH 6.8 and at 25°, showed adenosinetriphosphatase activity comparable to that for myosin B under the same condition.

From the above results, the protein in the supernatant solution obtained from the clear phase, is not myosin A, myosin B, actin, tropomyosin or any other protein fraction or protein complex obtained from muscle heretofore.

The molecules in the supernatant solution of 0.15 *M* KCl are very long but quite thin, and this filamentous protein seems to play an important role in the mechanism of superprecipitation. A possible and probable mechanism for the superprecipitation is as follows. The filamentous protein which is usually in a protected situation in the myosin B molecule or in the aggregated particle of myosin B, is liberated into the medium by the action of ATP accompanied by the dispersion of the rest of myosin B. This is the clear phase. After a while, the length of the interval depending upon the concentration of ATP, Mg^{++} *etc.*, the concentration of ATP is reduced by the adenosinetriphosphatase activity, and the filamentous protein molecules begin to attach themselves each other at various points, and a network is formed. The network of flexible chains would then shrink, picking up the dispersed myosin B molecules which now have larger tendency to attach to the filamentous protein because of the reduced concentration of ATP. The fact that the filamentous protein disaggregates into smaller molecules at a higher KCl concentration, is well in parallel with

the fact that superprecipitation takes place only in solutions of low KCl concentration.

Separation of a similar protein was suspected by the action of ATP in 0.6 *M* KCl, and a solution of myosin B in 0.6 *M* KCl was centrifuged at 100,000×*g* for two hours in the presence of ATP. The supernatant solution which was water clear and which was supposed to contain myosin A (*7*, *9*), was dialyzed against 0.15 *M* KCl solution. When the turbid solution was centrifuged to remove the aggregated myosin A, the supernatant solution showed birefringence indicating the presence of the filamentous protein. This protein was slightly different from that obtained from the clear phase of myosin B. The length calculated from the extinction angle was a little shorter, and the addition of ATP caused complete dissociation even in 0.15 *M* KCl solution. The amino acid composition was closer to that of myosin A, the ratio of Pro./Arg. being 0.6. The same kind of protein was obtained not by dialysing for the purpose of reduction of the ionic strength, but by diluting the 0.6 *M* KCl supernatant solution 4 times to make 0.15 *M*, and thus eliminating the possibility of denaturing the protein in the course of dialysis.

A detailed study of the nature and structure of the filamentous protein obtained in the present experiment is under way.

The author wishes to express his gratitude to Prof. S. Ebashi and Dr. F. Ebashi for their kind cooperation and helpful discussion.

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(Received for publication, June 9, 1960)

The Journal of Biochemistry, Vol. 48, No. 2, 1960

CONVERSION OF L-GULONOLACTONE TO L-ASCORBIC ACID; PROPERTIES OF THE MICROSOMAL ENZYME IN RAT LIVER

Recent reports by several workers have shown that the immediate precursor of L-ascorbic acid in animal tissue is L-gulonolactone and the enzyme capable of catalyzing the conversion of L-gulonolactone to L-ascorbic acid is present in rat liver microsomes (*1–3*). Kanfer *et al.* demonstrated that the microsomal enzyme possessed a configurational specificity for the laevo configuration of the C-2 hydroxyl group (*1*). Chatterjee *et al.* reported some properties (*3*) and cofactor of this enzyme (*4*). The present paper deals with further studies on the specificity of this enzyme, together with an account of some of its properties.

The assay conditions for the enzyme activity were as follows: the incubation was carried out in Warburg flasks containing 10 μmoles of substrate, microsomes prepared from 0.4 g of rat liver, and 150 μmoles of phosphate buffer (pH 7.0) in a final volume of 3.0 ml., in an atmosphere of O_2 for 30 minutes at 37° and the ascorbic acid formed was determined by the method of Roe and Kuether.

The effect of substrate concentration on the activity was examined under the usual assay conditions and results were plotted as shown in Fig. 1. The *Km* calculated from a Lineweaver-Burk plot was 2.5×10^{-3} *M* (Fig. 1).

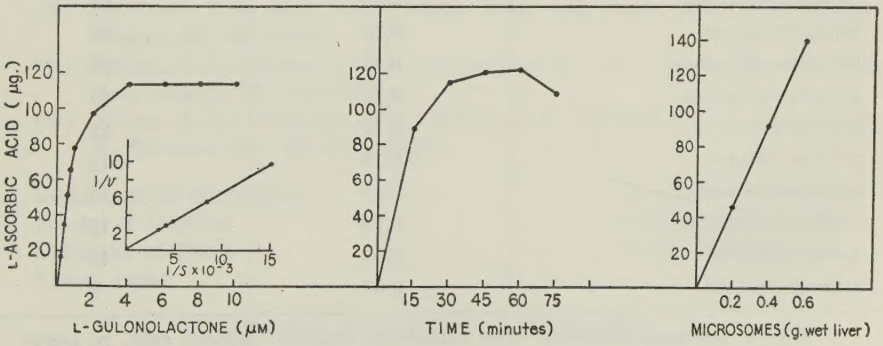

FIG. 1. Effect of substrate concentration on microsomal enzyme.[1] V is μmoles of ascorbic acid formed/30 minutes. S is L-gulonolactone in moles/liter.

FIG. 2. Effect of time on microsomal enzyme.[1]

FIG. 3. Effect of enzyme concentration of microsomal enzyme.[1]

1) The reaction conditions were indicated in the text.

The maximum formation of L-ascorbic acid took place after an incubation

period of 60 minutes (Fig. 2). Under the above conditions, the reaction was proportional to the enzyme concentration (Fig. 3).

There was some stimulation of activity of the microsomal enzyme with $\alpha\alpha'$-dipyridyl, EDTA, and 8-hydroxyquinoline (10^{-4} M each), and no inhibition occurred when sodium 5,5-diethylbarbiturate at 10^{-2} M (inhibitor of TPN- L-hexonate dehydrogenase*) or lycorine at 3.3×10^{-3} M were present in the incubation mixture.

In the absence of a suitable electron-acceptor there was no oxidation of L-gulonolactone by the microsomal enzyme under anaerobic condition. Various dyes were tested for their ability to act as electron-acceptor with this enzyme; these included Nile blue, triphenyltetrazolium chloride, Toluidine blue, Toluylene blue, and 2,6-dichlorophenol indophenol. Only the last one was reduced by the enzyme in the presence of a substrate.

The specificity of the enzyme was determined by assay of ascorbic acid or its analogues formed and estimation of oxygen consumption. The O_2 uptake agreed approximately with the value obtained by the method of Roe and Kuether. The result is given in Table I. As can be seen from this table, the microsomal enzyme appears to be specific for the laevo C-2 hydroxyl group of pentonic and hexonic acid except L-mannonolactone, L-lyxonolactone, and D-altronolactone. The free acids corresponding to the

TABLE I

Substrate Specificity of the Microsomal Enzyme[1]

substrate	μmole of L-ascorbic acid or analogue found	relative rate (L-gulonolactone = 100)
L-Gulonolatone	0.79	100
L-Galactonolactone	0.53	67
D-Lyxonolactone	0.36	46
D-Mannonolactone	0.27	34
D-Arabonoloactone	0.21	27
D-Idonolactone	0.17	22
D-Talonolactone	0.15	19
L-Gluconolactone	0.12	15
L-Mannonolactone	0.12	15
L-Xylonolactone	0.08	10
L-Lyxonolactone	0.07	9

All the following substances were completely inactive: glyceric acid, D, L-erythronic acid, L-threonolactone, D-ribonolactone, L-arabonolactone, D-xylonolactone, D-allonolactone, D-altronolactone, D-gluconolactone, D-gulonolactone, L-idonolactone, D-galactonolactone, D-α-glucoheptonolactone, D-β-glucoheptonolactone, L-rhamnonolactone and acids of all the above tested lactones.

1) The assay conditions were indicated in the text.

* in press (Mano, Y., Suzuki, K., Yamada, K., and Shimazono, N., *J. Biochem.*)

tested lactones and L-rhamnonolactone were completely inactive. These observations suggest that the lactone structure and the terminal alcohol group are essential for the activity. In general lactones which have the lactone ring of dextroposition* are more active than laevoposition.

Caputto *et al.* have reported that the enzymatic activity of the liver extract for the conversion of L-gulonolactone to L-ascorbic acid was significantly decreased in vitamin E deficient rats fed for 2–14 days (*5*, *6*). On the other hand, it was found in the present series of experiments that the rats fed on vitamin E deficient diet (*5*) for 45 days did not show any decrease of the activity of each of the enzymes which participate in the conversion of D-glucuronic acid to L-ascorbic acid. The restoration effect of tocopherol on the conversion of L-gulonolactone to L-ascorbic acid with the microsomes extracted by petroleum ether-ethanol (9 : 1) was recently reported by Chatterjee *et al.* (*4*), but no restoration was observed in the present experiments upon addition of tocopherol to the microsomes treated in the same way.

The activity for L-xylono- and L-lyxonolactone suggested that the enzyme might take part in the catabolism of L-ascorbic acid in animal tissue, because the free acids corresponding to these lactones are produced by the decarboxylation of 2,3-diketo-L-gulonic acid.**

This work was supported in part by a grant from the Waksman Foundation of Japan, Inc.

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(Received for publication, June 28, 1960)

* The position of the ring shows the former position of the hydroxyl group on the C-4.

** to be published (Y. Kagawa *et al.*)